Inhibition of human ornithine decarboxylase activity by enantiomers of difluoromethylornithine

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Racemic difluoromethylornithine (D/L-DFMO) is an inhibitor of ODC (ornithine decarboxylase), the first enzyme in eukaryotic polyamine biosynthesis. D/L-DFMO is an effective anti-parasitic agent and inhibitor of mammalian cell growth and development. Purified human ODC-catalysed ornithine decarboxylation is highly stereospecific. However, both DFMO enantiomers suppressed ODC activity in a time- and concentration-dependent manner. ODC activity failed to recover after treatment with either L- or D-DFMO and dialysis to remove free inhibitor. The inhibitor dissociation constant (K_D) values for the formation of enzyme–inhibitor complexes were 28.3 ± 3.4 , 1.3 ± 0.3 and 2.2 ± 0.4 μM respectively for D-, L- and D/L-DFMO. The differences in these K_D values were statistically significant ($P < 0.05$). The inhibitor inactivation constants (K_{inact}) for the irreversible step were 0.25 ± 0.03 , 0.15 ± 0.03 and 0.15 ± 0.03 min^{-1} respectively for D-, L- and D/L-DFMO. These latter values were not statistically significantly different ($P > 0.1$). D-DFMO was a more potent inhibitor ($\text{IC}_{50} \sim 7.5$ μM) when compared with D-ornithine ($\text{IC}_{50} \sim 1.5$ mM) of ODC-catalysed L-ornithine decarboxylation.

Treatment of human colon tumour-derived HCT116 cells with either L- or D-DFMO decreased the cellular polyamine contents in a concentration-dependent manner. These results show that both enantiomers of DFMO irreversibly inactivate ODC and suggest that this inactivation occurs by a common mechanism. Both enantiomers form enzyme–inhibitor complexes with ODC, but the probability of formation of these complexes is 20 times greater for L-DFMO when compared with D-DFMO. The rate of the irreversible reaction in ODC inactivation is similar for the L- and D-enantiomer. This unexpected similarity between DFMO enantiomers, in contrast with the high degree of stereospecificity of the substrate ornithine, appears to be due to the α -substituent of the inhibitor. The D-enantiomer may have advantages, such as decreased normal tissue toxicity, over L- or D/L-DFMO in some clinical applications.

Key words: difluoromethylornithine enantiomer, enzyme inactivation parameter, ornithine decarboxylase, polyamine.

INTRODUCTION

Polyamines are naturally occurring polycations derived from amino acids via decarboxylation reactions [1]. Genetic evidence indicates that polyamines are required for optimal growth of bacteria [2], and are essential for aerobic growth in yeast [3]. ODC (ornithine decarboxylase), the first enzyme in polyamine biosynthesis in eukaryotes, is essential for normal development in mammals. ODC gene knockouts are lethal in mouse embryos 3.5 days after fertilization [4].

ODC activity and polyamine contents are increased in many epithelial cancers [5]. ODC is a downstream transcriptional target for *c-myc* [6,7]. In a mouse model of intestinal carcinogenesis, mutation of the APC (adenomatous polyposis coli) tumour suppressor gene is associated with increased expression of ODC RNA and increased intestinal polyamine contents [8]. Increase of ODC expression in this mouse model apparently occurs because APC regulates the transcription of *c-myc* [9], which in turn regulates the ODC transcription [10]. Treatment of these mutant APC-expressing mice with D/L-DFMO (racemic difluoromethylornithine) suppresses intestinal carcinogenesis [8]. D/L-DFMO also inhibits carcinogenesis in chemical carcinogen-induced models of colon and other epithelial cancers, and is now in clinical trials in humans [5].

DFMO is also an effective anti-parasitic agent. This drug cures infection by *Trypanosoma brucei*, the causative agent in African sleeping sickness, in rodent models and humans [11,12]. DFMO is also active in the treatment of *Pneumocystis pneumonia* in AIDS patients [13].

Biochemical, genetic and structural evidence indicates that DFMO is bound at the active site of ODC, and irreversible inactivation involves formation of a reactive intermediate on decarboxylation of the inhibitor [14–16]. One preliminary report suggests that DFMO inhibition of ODC is non-stereospecific [17]. However, this study used a crude enzyme preparation and provided no evidence for non-stereospecific irreversible inactivation.

Despite the extensive information available for the effects of D/L-DFMO on cell and animal models, little is known about the stereospecific effects of this agent. A significant toxicity associated with high-dose (> 0.5 $\text{g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$) D/L-DFMO is usually reversible hearing loss [5,18]. In one single-dose study, L-DFMO, but not D-DFMO at the same dose, induced this ototoxicity in a guinea-pig model [19]. This study however did not determine whether the D-DFMO could produce ototoxicity at higher doses.

To gain further insight into the mechanism of stereospecific inhibition by DFMO, we first determined if both enantiomers were acting to inactivate ODC enzyme activity irreversibly, by using a

Abbreviations used: APC, adenomatous polyposis coli; BOC, t-butoxycarbonyl; DFMO, difluoromethylornithine; ODC, ornithine decarboxylase.

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highly purified recombinant His-tagged human ODC. Then, we measured the kinetic parameters for this inactivation. Finally, we measured the effects of DFMO enantiomers on cellular polyamine contents in a human colon tumour-derived cell line.

EXPERIMENTAL

Materials

L- and D-ornithine were purchased from Sigma. Purity of each ornithine enantiomer was 99 %. The enantiomers of DFMO were prepared from the racemate via crystallization and purification of a pair of diastereomeric derivatives. Briefly, D/L-DFMO was cyclized to the lactam form, which was converted in a multi-step reaction with optically pure (*R* or *S*) α -methyl benzamine to a pair of diastereomeric amide derivatives. The resulting diastereomers were resolved by fractional crystallization and purified by ion-exchange chromatography. Acidic hydrolysis of the pure diastereomers resulted in simultaneous cleavage of the amide groups and ring opening of the lactams to produce the optically pure DFMO enantiomers. Absolute configuration of the enantiomers was determined by X-ray crystallography. The optical rotations $[\alpha]$ of each of the DFMO enantiomers were measured and found to have the following values ($c = 0.5$ in methanol): -10° for the L-enantiomer and 7° for the D-enantiomer. As in the present case, when the specific rotations for molecules are not large, it is not unusual for the rotations to be opposite in sign and comparable in magnitude although not identical. The measurement is subject to slight variations in temperature, concentration and other variables.

An industrial-scale process was developed by NOVASEP (Boothwyn, PA, U.S.A.) for the purification of D/L-DFMO using multicolumn chromatography on the basis of VARICOL[®] [20]. D/L-DFMO was cyclized to form an *N*-pivaloyl DFMO lactam, which was chromatographically purified and each enantiomer was subsequently hydrolysed under acidic conditions (5.7 M HCl) to form pure enantiomers. A chiral analytical method was developed to monitor the potential racemization during ring opening and hydrolysis of the lactam. We were unable to develop a direct chiral separation method for D/L-DFMO. Therefore we employed BOC (t-butoxycarbonyl) chemistry to obtain the mono-BOC derivative of each of the DFMO enantiomers used in the present study. The source of the individual enantiomers used in the present study was obtained as enantiomerically pure external standards, which came from the lactams formed by the diastereomeric amide derivatives.

Mono-BOC derivatives were formed to confirm the enantiomeric purity of the D- and L-DFMO external samples. All reagents used in the synthesis and separation of the mono-BOC derivatives were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). In a 12 mm $\times$ 75 mm test tube, di-*t*-butyl dicarbonate (55 mg, 0.25 mM), either the D- or L-enantiomer of DFMO/HCl (50 mg, 0.21 mM), methanol (500 μ l) and triethylamine (60 μ l) were added. The suspension was shaken for 20 min. The resulting suspension was filtered through a 0.2 μ m syringe filter into a fresh test tube. A 10 mM solution of CuSO₄ (1 ml) was then added. The resulting solution was filtered and directly injected for chromatographic analysis. Evidence for the purity of the DFMO enantiomers, using chiral HPLC, is provided in the Results section.

L-[1-¹⁴C]Ornithine (47.7 mCi/mmol) was purchased from PerkinElmer Life Science (Boston, MA, U.S.A.). Restriction endonucleases were purchased from Invitrogen (Carlsbad, CA, U.S.A.). QIA expression and purification kits were purchased from Qiagen (Chatsworth, CA, U.S.A.). Other biochemical and chemical reagents were of analytical grade.

Preparation of human ODC protein

Plasmid DNA pODC was kindly provided by Dr Susan Gilmour (Lankenau Research Institute, Philadelphia, PA, U.S.A.). This plasmid contained a cDNA encoding full-length human ODC, as reported by Hickok et al. [21]. To express full-length human ODC by using QIA expression system, a 1.38 kb DNA fragment carrying the human ODC coding sequence was first amplified by PCR with pODC plasmid DNA as a template. The primers used for PCR were 5'-ATCGGGATCCATGAGCAGCTTT-3' (22mer) and 5'-CATTAGATCTAGCAGAAGCACAGGC-3' (25mer). These primers contained restriction sites for *Bam*HI and *Bgl*III (underlined), which were used to clone pODC as a C-terminal His₆-tag fusion that facilitates tight binding to Ni²⁺-nitrilotriacetate matrices. A 30-cycle PCR amplification was run for 1 min for the following temperatures: 94, 68 and 72 °C, with reaction mixtures as recommended by the methods accompanying the *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany) followed by a final additional extension at 72 °C for 10 min. The purified PCR product of pODC and PQE-60 expression vector (Qiagen), which contained a multiple clone site with C-terminal His₆-tag coding sequence, were digested with *Bam*HI and *Bgl*III. They were then ligated by T4 DNA ligase after the digested vector was dephosphorylated. Plasmids were transferred with *Escherichia coli* strain M15 competent cells that carried the pREP4 (Qiagen) repressor in the presence of both ampicillin (100 μ g/ml) and kanamycin (25 μ g/ml). The expression of pODC coding sequence was induced at the upstream Lac promoter by the addition of 2 mM of isopropyl β -D-thiogalactoside.

Purification of human ODC recombinant fusion protein under native conditions

To test the enzyme function and kinetic mechanism of human ODC, we purified pODC His₆ recombinant fusion protein by using the QIA purification system under native conditions. Luria–Bertani medium (100 ml) containing 100 μ g/ml ampicillin and 25 μ g/ml kanamycin was inoculated with an overnight culture. The culture was grown to attenuation D_{600} 0.5, and then induced with 2 mM isopropyl β -D-thiogalactoside. Growth was continued for an additional 4 h at 37 °C by shaking. Cells were collected by centrifugation and the pellet was resuspended in lysis buffer (50 mM NaH₂PO₄/300 mM NaCl/10 mM imidazole, pH 8.0). Cell membrane was broken with 1 mg/ml lysozyme for 30 min, and then sonicated on ice before centrifugation. The supernatant was mixed with 50 % Ni²⁺-nitrilotriacetate metal-affinity chromatography matrices (Qiagen) by stirring at 4 °C for 1 h. The mixture was loaded on to a chromatography column and washed with wash buffer (50 mM NaH₂PO₄/300 mM NaCl/20 mM imidazole, pH 8.0). The fusion protein was eluted with elution buffer (50 mM NaH₂PO₄/300 mM NaCl/250 mM imidazole, pH 8.0). The recombinant ODC protein purified in this single step had a specific activity of > 6 μ mol of L-ornithine decarboxylated/min per mg of enzyme protein. This is approx. 15 % of the specific activity of ODC purified from mouse kidney [22]. This difference could reflect differences in enzyme source or indicate that the recombinant protein has a lower specific activity than the native enzyme.

Protein determination and analysis

Protein concentrations were estimated by comparing the absorbance of a sample of unknown concentration with that of a sample of known BSA standard concentration in the same assay

by using the Bio-Rad reagent kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). SDS/PAGE was performed, as described elsewhere [10], to confirm the purity of the His-tagged ODC.

ODC activity and polyamine measurements

ODC activity was measured using two similar methods. In one method, described elsewhere by our group [23], the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine, during incubations for different times at 37 °C, was used to monitor ODC enzyme activity. One unit of ODC enzyme activity is defined as the amount of enzyme producing 1 nmol of $^{14}\text{CO}_2$ /min. The second method was identical with the first, except that non-radioactive L- or D-ornithine was used and decarboxylation was assessed by measurement of putrescine formation using HPLC. In this method, reactions were terminated by adjusting the reaction mixtures to 0.2 M HClO_4 before the separation of acid-soluble polyamines by the reversed-phase, ion-paired HPLC method described elsewhere [23,24].

For assessment of polyamine contents in cells, cultures were harvested, washed in PBS and disrupted by sonication in 0.1 M HCl at a concentration of 10^7 cells/ml. This sonicated suspension was then adjusted to 0.2 M HClO_4 , and acid-soluble polyamines were measured by HPLC as outlined above and elsewhere [23].

Cell culture

The human colon carcinoma cell line HCT116 was maintained in monolayer culture in McCoy's 5A medium, supplemented with 10 % (v/v) foetal bovine serum plus 1 % penicillin/streptomycin solution at 37 °C in a humidified atmosphere of 5 % CO_2 /95 % air. To measure the intracellular polyamine contents, HCT116 human colon cancer cells were seeded at 10^5 cells/ml in 20 ml of normal growth medium/T75 tissue culture flask in media supplemented with different concentrations of DFMO enantiomers (0.1, 0.5, 1 and 5 mM).

Statistical methods

Enzyme inactivation rates were determined by linear regression analyses using SPSS, v.10 (SPSS, Chicago, IL, U.S.A.). The enzyme inactivation parameters K_D and K_{inact} , as described by Kitz and Wilson [25], for DFMO enantiomers, were compared using the *t*-distribution on pooled estimated variances.

RESULTS

The chromatographic conditions for the separation of the enantiomers of D/L-mono-BOC DFMO and related enantiomers were obtained on a Chiralpak WH (Chiral Technologies, Exton, PA, U.S.A.) using a mobile phase of 10 mM CuSO_4 . The flow rate was 1.0 ml/min and the column temperature was 50 °C. The wavelength was 254 nm. The first eluting enantiomer was L-DFMO and the second was D-DFMO. Figure 1(A) shows the chromatographic profile of the separation of D/L-mono-BOC DFMO. Figure 1(B) shows the chromatographic profile of the first eluting enantiomer of L-DFMO. Given the limit of detection of this chiral assay, the excess enantiomer was estimated to be > 99 %. This confirms that racemization does not occur during the ring opening in acidic hydrolysis of the diastereomeric amide derivatives.

Additionally, we found the same results for the ring opening during acid hydrolysis of the D- or L-*N*-pivaloyl DFMO lactams. Acid hydrolysis takes place at 80 °C in 5.7 M HCl for more than 48 h. Figure 1(C) shows the chromatographic profile of the second eluting enantiomer of D-DFMO. As noted before, given the limit

Figure 1 Chiral HPLC analysis of mono-BOC derivatives of DFMO enantiomers

(A) Chromatographic profile of the separation of D/L-mono-BOC DFMO; (B) profile of the first eluting enantiomer of L-DFMO; and (C) chromatographic profile of the second eluting enantiomer of D-DFMO.

of detection of this chiral assay, the enantiomeric excess was estimated to be > 99 %.

To determine the mechanism of inhibition of the DFMO stereoisomers, purified human ODC was incubated with different concentrations of L- and D-DFMO for 30 min. Samples were then divided into two parts. One part was immediately assayed for ODC enzyme activity. The other part was dialysed for 48 h against two changes of ODC reaction buffer. After dialysis, the second part was assayed for ODC enzyme activity. Essentially, all the ODC enzyme activity was recovered after dialysis in samples not treated with DFMO (Figure 2). For a given concentration, L-DFMO was more potent compared with D-DFMO in suppressing the ODC activity, but both stereoisomers inhibited ODC in a concentration-dependent manner. ODC activity did not recover after dialysis, following treatment with either of the stereoisomers.

Mechanisms of inactivation of ODC by DFMO were investigated further by performing time- and concentration-dependent inhibition studies of purified human His-tagged ODC (Figure 3). The enzyme (2 μg) was incubated for periods up to 60 min with different concentrations of DFMO enantiomers, and then the enzyme activity was determined by adding radiolabelled L-ornithine for 3 min. All DFMO forms decreased ODC enzyme activity with near first-order kinetics, from which an apparent rate constant (K_{app}) was estimated. For given concentrations of DFMO, the L-enantiomer was more effective than the D-enantiomer in

Figure 2 Effect of dialysis on ODC activity treated with or without DFMO enantiomers in purified human ODC

Purified ODC enzyme (25 μ g) was incubated with or without 10 μ M of DFMO enantiomers for 30 min. Samples were then divided into two portions and taken in two separate tubes. Enzyme activity was determined immediately in one sample (black bars). Another sample was exhaustively dialysed for 48 h (grey bars), with three changes of ODC assay buffer not containing inhibitors. Enzyme activity was then determined after dialysis.

Figure 3 Inhibition of ODC activity by DFMO enantiomers in purified human ODC

Purified ODC protein was incubated at various concentrations ($\circ$, 0.5; $\blacksquare$, 1; $\square$, 2; $\blacktriangle$, 5; $\triangle$, 10 μ M) of DFMO enantiomers for different time periods at 37 $^{\circ}$ C. The inhibitor was always present during the period of $^{14}\text{CO}_2$ release. Percentage control ODC enzyme activity was then determined by dividing the ODC enzyme activity for each incubation interval before enzyme activity determination, as shown in the Figure, by the activity of ODC enzyme incubated with inhibitor only during the period of $^{14}\text{CO}_2$ release. Time-dependent inhibition of ODC by different concentrations of DFMO enantiomers is depicted for L-DFMO (A), D/L-DFMO (B) and D-DFMO (C).

decreasing the ODC enzyme activity. The D/L-DFMO was intermediate, compared with the L- and D-enantiomer, in decreasing the ODC enzyme activity.

The reaction scheme for the inactivation of ODC by DFMO enantiomers is

where $K_D = [\text{ODC enzyme}][\text{DFMO}]/[\text{ODC enzyme-inhibitor complex}]$ is the inhibitor dissociation constant. To determine the values of K_D and inhibitor inactivation constant (K_{inact}), reciprocal K_{app} was plotted as a function of the reciprocal of

Figure 4 Double-reciprocal plots of K_{app}^{-1} as a function of reciprocal inhibitor concentrations

Values of K_{app} were determined from results shown in Figure 3. Variation of K_{app}^{-1} as a function of the inverse concentration of inhibitors ($\bullet$, L-DFMO; $\blacksquare$, D/L-DFMO; $\blacktriangle$, D-DFMO) is shown and was used to compute kinetic constants (see Table 1).

Table 1 Kinetic parameters

(a) Kinetic constants for DFMO enantiomers, as determined from time- and concentration-dependent inhibition studies shown in Figures 2 and 3 and (b) comparison of K_{inact} and K_D values.

	K_{inact} (min^{-1})	S.E.M.	K_D (μM)	S.E.M.
L-DFMO	0.15	± 0.03	1.3	± 0.3
D-DFMO	0.25	± 0.03	28.3	± 3.4
D/L-DFMO	0.15	± 0.03	2.2	± 0.4

(b)

K_{inact}	K_D
L = D ($P = 0.22$)	L $\neq$ D ($P < 0.001$)
L = DL ($P > 0.5$)	L $\neq$ DL ($P = 0.04$)
D = DL ($P = 0.16$)	D $\neq$ DL ($P < 0.001$)

the inhibitor concentration [25] (Figure 4). The resulting kinetic parameters are summarized in Table 1. This analysis indicates that the K_D for the formation of ODC enzyme-inhibitor complexes is 20 times greater for the D-enantiomer when compared with the L-enantiomer and this difference is statistically significant ($P < 0.001$). This suggests that fewer enzyme-inhibitor complexes are formed, for a given amount of enzyme and inhibitor in the case of the D-enantiomer, relative to the L-enantiomer. The K_D for D/L-DFMO is nearly twice that for L-DFMO, consistent with the large differences in affinity for the L- and D-enantiomers. The K_{inact} for the irreversible inactivation of ODC is not, or only marginally, stereospecific. This indicates that the L- and D-enantiomers of DFMO, once complexed with ODC, are decarboxylated by the enzyme at similar rates.

The results indicating that D-DFMO irreversibly inactivated ODC enzyme activity were surprising, considering the high degree of stereospecificity of ODC as an L-amino acid decarboxylase. To substantiate these findings, we first compared the ability of human ODC with decarboxylate L- and D-ornithines. The human ODC preparation employed had a specific activity of $> 6 \mu\text{mol}$ of L-ornithine decarboxylated/min per mg of protein. Using $> 99\%$ pure D-ornithine, human ODC decarboxylated D-ornithine, albeit poorly, in a time-dependent manner. Incubation of ODC with 200 μM D-ornithine for either 20 or 30 h converted

Figure 5 Comparison of D-ornithine and D-DFMO as inhibitors of L-ornithine decarboxylation by human ODC

Purified ODC enzyme (2 μg) was incubated for 30 min at 37 °C in the presence of different concentrations of D-DFMO (●) and D-ornithine (■) before the addition of radiolabelled L-ornithine for enzyme activity assay. The mean and S.E. were calculated from three independent experiments.

the substrate to putrescine by 2.2 and 5.3% respectively. Thus ODC could decarboxylate D-ornithine to yield authentic putrescine, as measured by HPLC, but the specific activity of the enzyme using this substrate was <0.0001 μmol of D-ornithine decarboxylated/min per mg of enzyme protein. Thus both the ornithine enantiomers were decarboxylated by ODC, but the specific activity was 10000 times greater for L-ornithine when compared with D-ornithine.

We then compared D-ornithine and D-DFMO as inhibitors of L-ornithine decarboxylation by ODC. In terms of concentration, D-DFMO was >100 times more effective than D-ornithine as an inhibitor of ODC activity, using L-ornithine as a substrate (Figure 5). Together, these results indicate that ODC can decarboxylate D-ornithine and that D-DFMO is a more potent inhibitor of ODC than D-ornithine. The implications of these results for a plausible mechanism of ODC inactivation by D-DFMO will be discussed in the Discussion section.

The physiological significance of the differences in suppression of ODC by stereoisomers of DFMO was confirmed by treating human HCT116 colon tumour cells with DFMO enantiomers (Figure 6). High doses of both L- and D-enantiomer suppressed putrescine and spermidine, but not spermine, contents. However, suppression of spermidine contents was stereospecific over the concentration range 0.1–1 mM for 24 h treatments.

DISCUSSION

DFMO was developed more than two decades ago, and is now widely used in both experimental studies [1] and clinical applications, including cancer prevention [5], treatment of parasitic diseases [11,13] and hair removal [26]. D/L-DFMO has shown to be an enzyme-activated, irreversible inhibitor of ODC [27]. Biochemical mechanisms for the inactivation of ODC by DFMO have been proposed [14], and genetic studies have corroborated many features of this model [16]. Trypanosomal ODC–DFMO complexes have been crystallized, and subsequent structural analysis has added to our understanding of the mechanism of this specific enzyme inhibition [22]. No biochemical, genetic or structural studies have investigated the mechanism of ODC inactivation by specific stereoisomers of DFMO.

Results presented here indicate that both the L- and D-forms of DFMO are potent and irreversible inhibitors of human ODC.

Figure 6 Effects of enantiomers on polyamine contents in HCT116 cells

HCT116 human colon cells were grown in the absence or presence of different concentrations of DFMO enantiomers (0.1, 0.5, 1 and 5 mM) for 24 h. Cultures were harvested and acid-soluble polyamine and acid-insoluble protein contents were determined as indicated in the Experimental section (♦, D-DFMO; ■, L-DFMO; ▲, D/L-DFMO). Put, putrescine; Spd, spermidine; Spm, spermine.

The L-enantiomer, however, is a more effective inhibitor of ODC compared with the D-enantiomer. The difference in activity between the two enantiomers appears to be due to differences in the formation of the ODC enzyme–DFMO-inhibitor complexes. This conclusion is based on the measurement of the equilibrium constant K_D for this reaction, and suggests that L-DFMO forms these complexes >20 times more readily than D-DFMO. The rate of irreversible enzyme inactivation, K_{inact} , is similar for the L- and D-forms of this inhibitor.

Results of the present study suggest that both D- and L-DFMO irreversibly inactivate ODC by a similar mechanism in a manner described earlier for D/L-DFMO [14–16]. The finding that D-DFMO irreversibly inactivated ODC was initially surprising, in light of the high degree of stereospecificity of the substrate ornithine (ODC specific activity different by a factor of 10000 for L- and D-enantiomer). D-DFMO is 100 times more active when compared with D-ornithine, as an inhibitor of L-ornithine decarboxylation. These results suggest a mechanism in which the α -substituent of D-DFMO increases the formation of enzyme–inhibitor complexes when compared with the association of D-ornithine at the active site of ODC. Once these enzyme–inhibitor complexes are formed, the rate of decarboxylation of either inhibitor or substrate is only marginally stereospecific. Previous studies [17] have proposed that the α -substitution in ornithine analogues may induce a misplacement of the pyridoxal-phosphate cofactor–inhibitor complex in the enzyme active site, thus rendering analogues like D-DFMO better substrates for ODC when compared with D-ornithine.

Treatment of cultured human cells with the enantiomers corroborate the general conclusion that both enantiomers are potent inhibitors of ODC, but that the L-DFMO is more active than the D-DFMO. One previous study reported that L-DFMO, but not D-DFMO, induced ototoxicity in a guinea-pig model [19]. Ototoxicity is the dose-limiting toxicity in many cancer-prevention and -treatment trials [5,12]. The results of the present study indicate that D-DFMO may also induce this effect in animal models and in humans, but higher doses of the D-form of the inhibitor may be required, compared with the L- or D/L-forms of DFMO to produce either therapeutic or toxic reactions.

The kinetic differences in inactivation of ODC by enantiomers of DFMO may have clinical applications. D/L-DFMO is an effective inhibitor of ODC when it can be delivered in an adequate concentration to maintain levels of ODC enzyme-inhibitor complexes for sufficient durations for the irreversible inactivation step to occur. Enantiomers of DFMO may be useful in combination with other strategies such as tissue-specific delivery of DFMO by topical and regional applications [28,29] or dose de-escalation [30] to optimize treatment strategies using DFMO.

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