Inhibition of human ornithine decarboxylase activity by enantiomers of difluoromethylornithine

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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 g·m⁻²·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 otoxicity in a guinea-pig model [19]. This study however did not determine whether the D-DFMO could produce otoxicity 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
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 [α] 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 VARI-COL® [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 samples. The starting material was derived 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 × 75 mm test tube, di-tert-butyl dicarbonate (55 mg, 0.25 mM), either the D- or L-enantiomer of DFMO/HCl (50 mg, 0.21 mM), methanol (500 µl) and triethylamine (60 µl) were added. The suspension was shaken for 20 min. The resulting suspension was filtered through a 0.2 µm 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′-ATCCGGGATCCATTGACACGTTT-3′ (22mer) and 5′-CATTGAATTCGAGAAGCCACAGGC-3′ (25mer). These primers contained restriction sites for BanHI and BglII (underlined), which were used to clone pODC as a C-terminal His-tag fusion that facilitates tight binding to Ni2+–nitrilotriacetic 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) for the purification of D/L-DFMO using QIA expression and purification kits purchased from Qiagen. These plasmids were transferred with *Escherichia coli* strain M15 competent cells that carried the pREP4 (Qiagen) repressor in the presence of both ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

The expression of pODC coding sequence was induced 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.uria–Bertani medium (100 ml) containing 100 µg/ml ampicillin and 25 µg/ml kanamycin was inoculated with an overnight culture. The culture was grown to attenuation $D_{A_{260}}$ 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+-nitrilotriacetic 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 µmol 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.

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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 $^{14}$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 $^{14}$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$^5$ 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 $K_a$ and $K_{	ext{app}}$, 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 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 ($K_{	ext{app}}$) 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 µg) was incubated with or without 10 µM 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 (0.5, 1; □, 2; ▲, 5; △, 10 µM) of DFMO enantiomers for different time periods at 37 °C. The inhibitor was always present during the period of 14CO2 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 14CO2 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

\[ \text{ODC enzyme} + \text{DFMO} \rightleftharpoons \text{ODC–inhibitor complex} \]

\[ \text{K}_{\text{D}} \rightleftharpoons \text{irreversibly inactivated ODC} \]

where \[ K_{\text{D}} = [\text{ODC enzyme}][\text{DFMO}]/[\text{ODC enzyme–inhibitor complex}] \] is the inhibitor dissociation constant. To determine the values of \( K_{\text{D}} \) and inhibitor inactivation constant (\( K_{\text{inact}} \)), reciprocal \( K_{\text{app}} \) was plotted as a function of the reciprocal of the inhibitor concentration [25] (Figure 4). The resulting kinetic parameters are summarized in Table 1. This analysis indicates that the \( K_{\text{D}} \) 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 \( K_{\text{D}} \) 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 \( K_{\text{inact}} \) 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 µmol 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...
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.

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 $K_c$ for this reaction, and suggests that L-DFMO forms these complexes >20 times more readily than D-DFMO. The rate of irreversible enzyme inactivation, $K_{max}$, 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 corroborates 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 complex 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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