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DL-α-Monofluoromethylputrescine is a potent irreversible inhibitor of *Escherichia coli* ornithine decarboxylase

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DL-α-Monofluoromethylputrescine (compound R.M.I. 71864) is an enzyme-activated irreversible inhibitor of the biosynthetic enzyme ornithine decarboxylase from *Escherichia coli*. This compound, however, has much less effect *in vitro* on ornithine decarboxylase obtained from *Pseudomonas aeruginosa*. These findings are in contrast with those previously found with the substrate analogue DL-α-difluoromethylornithine (compound R.M.I. 71782). The K_i of the DL-α-monofluoromethylputrescine for the *E. coli* ornithine decarboxylase is 110 μM, and the half-life ($t_{\frac{1}{2}}$) calculated for an infinite concentration of inhibitor is 2.1 min. When DL-α-monofluoromethylputrescine is used in combination with DL-α-difluoromethylarginine (R.M.I. 71897), an irreversible inhibitor of arginine decarboxylase, *in vivo* in *E. coli*, both decarboxylase activities are inhibited (>95%) but putrescine levels are only decreased to about one-third of control values and spermidine levels are slightly increased.

ODC (EC 4.1.1.17) catalyses the decarboxylation of ornithine to putrescine, the ubiquitous diamine found in all organisms studied to date (Pegg & Williams-Ashman, 1981). In attempts to elucidate the role of putrescine and higher polyamines formed from putrescine, several ODC inhibitors have been prepared (Jänne & Heby, 1981). An elegant approach to specific irreversible inactivation of ODC has been to design inhibitors possessing latent reactive groups that are unmasked at the enzyme active site as a result of normal catalytic turnover (Rando, 1974; Seiler *et al.*, 1978*b*). The substrate analogue DFMO (compound R.M.I. 71782) is one of the most useful enzyme-activated irreversible ODC inhibitors and has been studied in various mammalian and non-mammalian eukaryotic systems *in vitro* (Metcalf *et al.*, 1978), in cell culture (Mamont *et al.*, 1978) and *in vivo* (Koch-Weser *et al.*, 1981; McCann *et al.*, 1981). It has also been shown that some product analogues, namely hex-5-yne-1,4-diamine ('DL-α-acetylenicputrescine'; compound R.M.I. 71696) and *trans*-hex-2-en-5-yne-1,4-diamine ('DL-α-acetylenicdehydroputrescine'; compound R.M.I. 71873), are irreversible inhibitors of mammalian ODC and that the mechanism of the inactivation demands catalytic turnover (Metcalf *et al.*, 1978). In addition, injection of another product analogue, 1,4-diamino-5-fluoropentane ('DL-α-monofluoromethylputrescine'; compound R.M.I. 71864), into rats causes an inhibition of ODC activity in the ventral prostate and in thymus (Seiler *et al.*, 1978*a*).

In a previous study we found that DFMO caused an irreversible inhibition of ODC obtained from *Pseudomonas aeruginosa* but had no effect *in vitro* on ODC obtained from the Enterobacteria *Escherichia coli* and *Klebsiella pneumoniae* (Kallio & McCann, 1981), indicating that there are some differences of specificity for ODC inhibitors in these bacterial enzymes. Thus the possibility existed that some analogue of ornithine or putrescine might be an irreversible inhibitor of an ODC from Enterobacteria, but without effect on the enzyme from *Pseudomonas aeruginosa*. In the present study we report that DL-α-monofluoromethylputrescine is a potent irreversible inhibitor of ODC in *E. coli* but is much less effective *in vitro* on ODC activity of *Pseudomonas aeruginosa*.

Abbreviations used: ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine.

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Experimental

Chemicals

DL-[1-^{14}C]Ornithine (sp. radioactivity 58 Ci/mol) was purchased from The Radiochemical Centre

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(Amersham, Bucks., U.K.). DL-[1-^{14}C]Arginine (sp. radioactivity 20Ci/mol) was purchased from C.E.A. Research Products International (Mt. Prospect, IL, U.S.A.) and *S*-adenosyl-L-[1-^{14}C]methionine (sp. radioactivity 60Ci/mol) was purchased from New England Nuclear (Boston, MA, U.S.A.). The acetylenicputrescine analogues (Metcalf *et al.*, 1978) DFMO and DL-α-difluoromethylarginine (Bey *et al.*, 1979) and DL-α-monofluoromethylputrescine (P. Bey, unpublished work) were synthesized at the Centre de Recherche Merrell International. All other reagents were analytical grade.

Preparation of enzyme extracts

E. coli (strain M.R.C. 59), *Pseudomonas aeruginosa* (type A.T.C.C. 9027) and *Klebsiella pneumoniae* (type A.T.C.C. 8045) were grown in Davis & Mingioli (1950) media at 37°C with vigorous aeration up to late exponential–early stationary phase. Cells were washed once with phosphate-buffered saline, pH 7.2 (0.3M-NaCl/10mM-Na_2HPO_4/3mM-KH_2PO_4) and resuspended in 10mM-Tris/HCl buffer, pH 7.5, containing 1mM-dithiothreitol and 0.1mM-EDTA. After sonication in a Branson Cell Disrupter 350 set at a power setting of 2 for 2min the cell debris was removed by centrifugation at 20000*g* for 30min. The supernatant was used for enzyme activity measurements.

Time-dependency of the inhibition

Enzyme preparation (60μl) was mixed with 60μl of 300mM-Tris/HCl, pH 8.25, containing 3mM-dithiothreitol and 0.16mM-pyridoxal phosphate. Of this mixture 20μl was removed for activity measurements then 50μl of an aqueous solution of inhibitor was added. At various times 20μl portions of this solution were transferred to the final assay mixture containing the substrate, resulting in a 50-fold dilution of the inhibitor. This transfer effectively stopped any further enzyme inhibition.

Assay for enzyme activities

Biosynthetic ODC and arginine decarboxylase activities were measured as previously described (Kallio & McCann, 1981) using 10mM-L-ornithine and 0.5mM-L-arginine concentrations in the assays. *S*-Adenosylmethionine decarboxylase activity was assayed in 100mM-sodium phosphate buffer, pH 7.4, containing 20mM-$MgSO_4$, 3mM-dithiothreitol and 0.4mM-substrate (Jänne & Williams-Ashman, 1971). The incubations were carried out for 20min at 37°C. Protein concentrations were determined by the method of Bradford (1976) with crystalline bovine serum albumin as standard.

Assay for polyamines

E. coli cells were harvested rapidly by centrifugation, washed once with phosphate-buffered saline, pH 7.2, and extracted with 0.4ml of 40% trichloroacetic acid. Proteins were removed by centrifugation and the supernatant was filtered with a 0.45μm Millipore filter. Portions (20μl) of supernatant were analysed for polyamines with a Dionex D-300 Polyamine Analyzer using a Dionex P/N 30831 cation-separator column followed by post-column 'derivatization' with *o*-phthalaldehyde and fluorescence detection. The following buffers were used: for equilibration and putrescine elution, 0.3M-sodium citrate buffer, pH 5.8 (sodium citrate, 0.98g/litre; NaCl, 16.94g/litre; phenol, 1ml/litre); for spermidine elution, 0.7M-sodium citrate buffer, pH 5.55 (sodium citrate, 1.97g/litre; NaCl, 42.63g/litre; phenol 1ml/litre). The eluent flow rate was 36ml/h; reagent (*o*-phthalaldehyde) flow rate was 18ml/h and the column temperature was 45°C. DL-α-Monofluoromethylputrescine eluates after putrescine on the column and does not interfere with the putrescine analysis.

Results

Inhibition of bacterial ODC activity by product and substrate analogues

Each of the three putrescine analogues tested inhibited biosynthetic ODC obtained from *E. coli in vitro* (Table 1). DL-α-Difluoromethylornithine, as shown previously (Kallio & McCann, 1981), had no effect. The inhibition was tested by pre-incubating the crude enzyme extract in the presence of 1.25mM test compound in the assay mixture without substrate. After 15min L-ornithine was added, adjusting the inhibitor and substrate concentrations to 1mM and 10mM respectively, and the mixture was incubated for an additional 20min. DL-α-Monofluoromethylputrescine was the most potent inhibitor of the biosynthetic *E. coli* ODC, inhibiting the enzyme activity by more than 99% (Table 1). Hex-5-yne-1,4-diamine and *trans*-hex-2-en-5-yne-1,4-diamine showed 46% and 69% inhibition of *E.*

Table 1. *Effect of various putrescine and ornithine analogues on E. coli ODC activity in vitro*

The inhibition of the enzyme activity was measured by pre-incubating the enzyme with the compound (1.25mM) at 37°C in assay mixture lacking ornithine. After 15min L-ornithine was added and enzyme activity was measured.

Compound	Inhibition (%)
DL-α-Monofluoromethylputrescine	>99
trans-Hex-2-en-5-yne-1,4-diamine	69
Hex-5-yne-1,4-diamine	46
DFMO	0

coli ODC activity respectively (Table 1). Under identical conditions, DL-α-monofluoromethylputrescine caused a 50% inhibition of ODC obtained from *Klebsiella pneumoniae* and only a 15% inhibition of the ODC obtained from *Pseudomonas aeruginosa*. This was a reversal of the species specificity seen when DFMO was tested against different bacterial ODCs (Kallio & McCann, 1981).

Time-dependency of monofluoromethylputrescine inhibition of E. coli ODC

Progressive enzyme inhibition with time is usually taken as strong evidence for irreversible enzyme inhibition (Rando, 1974; Abeles & Maycock, 1976; Walsh, 1977). *E. coli* ODC activity decreased with apparent first-order kinetics when incubated with DL-α-monofluoromethylputrescine. The inactivation process was followed for at least two half-lives in the presence of inhibitor concentrations ranging from 0.025 to 0.2 mM. The apparent dissociation constant (K_i) was determined to be 110 μM by plotting the half-lives of the enzyme activities as a function of the inverse of inhibitor concentration (Kitz & Wilson, 1962; Jung & Metcalf, 1975) as shown in Fig. 1. The half-life of the enzyme at 10 μM inhibitor concentration was 25 min and the half-life at an infinite concentration of inhibitor was calculated to be 2.1 min. The presence of dithiothreitol in the pre-incubation mixture and the absence of a lag time before the onset of inhibition is evidence against the possibility of inhibition via an affinity-labelling mode by a diffusable alkylating species. Dialysis of the completely inactivated enzyme against 50 mM-Tris/HCl buffer, pH 8.25, containing 1 mM-dithiothreitol and 0.1 mM-EDTA for 24 h failed to restore enzyme activity to more than 3% of the initial amount, suggesting that the inhibitor formed a stable adduct with the enzyme. The addition of 2 mM-L-ornithine to the pre-incubation mixture partially protected the enzyme against inhibition by DL-α-monofluoromethylputrescine, indicating that the inhibition was active-site-directed (Metcalf *et al.*, 1978).

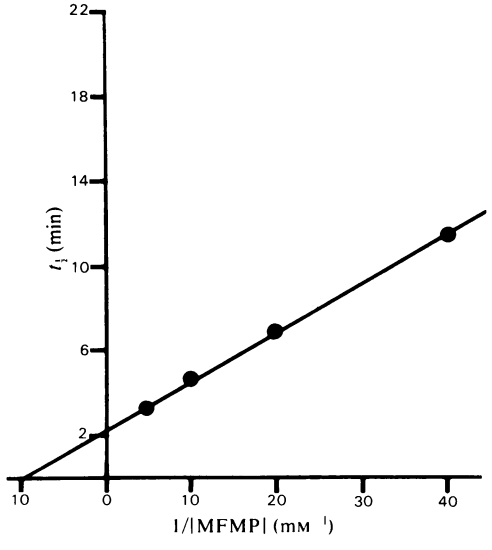

Fig. 1. *The dependence of the half-life of E. coli biosynthetic ODC on the concentration of* DL-α-*monofluoromethylputrescine* (*MFMP*)

The half-life of the enzyme activity at different concentrations of DL-α-monofluoromethylputrescine (0.025–0.2 mM) was determined as described in the Experimental section.

Effect of monofluoromethylputrescine in vivo on polyamine biosynthetic enzymes and polyamine levels in E. coli

To determine if DL-α-monofluoromethylputrescine would inhibit ODC activity in dividing cells, *E. coli* was grown in media supplemented with 1 mM-DL-α-monofluoromethylputrescine or 1 mM-DL-α-difluoromethylarginine (compound R.M.I. 71897), a potent irreversible arginine decarboxylase inhibitor (Kallio *et al.*, 1981), or with a combination of the two. After 4 h, when the cells were in the early stationary phase of growth, cells were harvested, washed with phosphate-buffered saline and sonicated in 10 mM-Tris/HCl, pH 7.5, containing 1 mM-dithiothreitol and 0.1 mM-EDTA. As shown in Table 2, DL-α-monofluoromethylputrescine caused a 95% inhibition of ODC activity in *E. coli* and moderately stimulated arginine decarboxylase and *S*-adenosylmethionine decarboxylase, other enzymes involved in polyamine biosynthesis. The combination of DL-α-monofluoromethylputrescine and DL-α-difluoromethylarginine almost totally abolished the activities of ODC and arginine decarboxylase in *E. coli* cells, whereas the activity of *S*-adenosylmethionine decarboxylase was enhanced by 30%. Dialysis of the extracts for 20 h at +4°C in 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol and 0.1 mM-EDTA, did not affect the inhibition or the stimulation of the enzyme activities.

The effect of these inhibitors on polyamine levels was determined by analysing extracts from *E. coli* grown in the presence of 1 mM-monofluoromethylputrescine, 1 mM-DL-α-difluoromethylarginine or a combination of the two, each at 1 mM. After 4 h the combination of inhibitors caused a 65% decrease in the putrescine levels (Table 3). Neither compound alone had any effect on the putrescine concentration. Spermidine levels were increased 60% when the inhibitors were used in combination (Table 3). ODC and/or arginine decarboxylase activities were inhibited 85–95% in these 4 h experiments. In a further experiment where the concentrations of DL-α-monofluoromethylputrescine and DL-α-di-

Table 2. *Effect in vivo of analogues on ODC activities of E. coli*

E. coli was grown in Davis & Mingioli (1950) media in the absence or presence of 1 mM-DL-α-difluoromethylarginine (DFMA), DL-α-monofluoromethylputrescine (MFMP) or a combination of these two inhibitors. After 4 h, cells were harvested and enzyme activities were measured as described in the Experimental section.

Compound	ODC activity (μmol/h per mg of protein)	Arginine decarboxylase (μmol/h per mg of protein)	*S*-Adenosylmethionine decarboxylase (μmol/h per mg of protein)
None	0.97	0.64	0.20
DFMA	1.28	0.03	0.21
MFMP	0.05	0.73	0.26
DFMA + MFMP	0.06	0.05	0.26

Table 3. *Effect in vivo of* DL-α-*monofluoromethylputrescine (MFMP),* DL-α-*difluoromethylarginine (DFMA) or their combination on polyamine levels of E. coli*

E. coli was grown as detailed in Table 2. Numbers of cells at initiation of experiment = $(2.43 \pm 0.07) \times 10^8$ cells/ml; in controls and all experimental groups after 4 h, numbers of cells = $(19.9 \pm 0.09) \times 10^8$ cells/ml. Results are means (±S.D.) from four experiments.

Compound	Putrescine (nmol/10^8 cells)	Spermidine (nmol/10^8 cells)
None	1.58 ± 0.15	0.34 ± 0.06
MFMP (1.0 mM)	1.63 ± 0.13	0.33 ± 0.05
MFMP (2.5 mM)	1.29 ± 0.16	0.36 ± 0.06
DFMA (1.0 mM)	1.45 ± 0.26	0.33 ± 0.04
DFMA (2.5 mM)	1.18 ± 0.15	0.42 ± 0.08
MFMP + DFMA (both 1.0 mM)	0.54 ± 0.12	0.46 ± 0.06
MFMP + DFMA (both 2.5 mM)	0.44 ± 0.01	0.48 ± 0.07

fluoromethylarginine were each increased to 2.5 mM, the putrescine level was still only partially decreased (72% inhibition) after growing the cells for 4 h in the presence of both inhibitors (Table 3). At the 2.5 mM concentration, however, both inhibitors alone did cause a moderate decrease in the intracellular putrescine levels, i.e. a maximum of 25% (Table 3). At neither concentration, 1.0 mM or 2.5 mM, did either inhibitor or the combination thereof have any effect on the growth rate of cells after 4 h (Table 3) or even after 8 h of incubation (results not shown).

Discussion

The product analogue DL-α-monofluoromethylputrescine irreversibly inactivates ODC from *E. coli* in an example of the microscopic-reversibility principle (Metcalf *et al.*, 1978) but has little or no effect on ODC in crude extracts of *Pseudomonas aeruginosa*. This is in contrast with the findings with the substrate analogue DFMO, which irreversibly inhibits all *Pseudomonas* ODC activity in the same crude extracts but has no effect on the enzyme from *E. coli* (Kallio & McCann, 1981). Of the various eukaryotic ODCs that have been tested, all are irreversibly inhibited by DL-α-difluoromethylornithine (Seiler *et al.*, 1978*a*; Koch-Weser *et al.*, 1981; McCann *et al.*, 1981). DL-α-Monofluoromethylputrescine has not been widely used but it has been shown to be an effective irreversible inhibitor of several mammalian ODCs in various tissues (Seiler *et al.*, 1978*a*). Thus, there seems to be a true difference between the two bacterial species with regard to specificity for the two analogues, both of which irreversibly bind mammalian ODCs. This difference in specificity is probably not related to whether the inhibitor is a substrate or product analogue, and in both cases detailed studies have to be done to determine the specific chemical basis of the irreversible inhibition.

It is not clear why putrescine levels were only decreased by about 70% when both DL-α-monofluoromethylputrescine and DL-α-difluoromethylarginine were used in combination, whereas arginine decarboxylase and ODC activities were inhibited up to 95%. It could be due to the residual decarboxylase activities preventing any further decrease in putrescine, or it may reflect changes in putrescine metabolism or possibly alternate routes of biosynthesis. Another possibility is that transport of the

inhibitors into the cells is not very rapid and by 4h maximal inhibition of the two decarboxylase activities is achieved before a concomitant effect on putrescine levels.

The combination of two inhibitors of putrescine biosynthesis will significantly reduce *E. coli* putrescine levels, but even after 8h will not have any effect on the growth rate of the cells. Similar results were noted with *Pseudomonas aeruginosa* when DFMO and difluoromethylarginine were combined (Kallio & McCann, 1981). As Table 3 indicates, however, spermidine levels seem to have actually increased when the two inhibitors were used in combination.

Finally, the present data and previous results with DFMO (Kallio & McCann, 1981) indicate that the effect of any potential inhibitor on ODC from bacteria cannot be predicted on the basis of action against eukaryotic enzymes. A similar example to this has been reported with glutamate decarboxylase, another pyridoxal phosphate-linked enzyme, wherein α-fluoromethylglutamic acid irreversibly inhibited the *E. coli* enzyme but had little effect on the mammalian brain enzyme (Kuo & Rando, 1981). Thus, the possibility seems to exist that a compound could have a specificity for bacterial ODC without having any effect on the mammalian enzymes.

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