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(2R,5R)-6-HEPTYNE-2,5-DIAMINE, AN EXTREMELY POTENT INHIBITOR OF MAMMALIAN ORNITHINE DECARBOXYLASE

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Summary: It was previously shown that 5-hexyne-1,4-diamine is a potent enzyme-activated irreversible inhibitor of mammalian ornithine decarboxylase. However this compound has secondary pharmacological effects owing to its in vivo oxidation to 4-aminohex-5-ynoic acid, an irreversible inhibitor of 4-aminobutyrate aminotransferase. The first step of this oxidation is catalysed by mitochondrial monoamine oxidase. The monomethyl and dimethyl analogues of 5-hexyne-1,4-diamine, i.e. 6-heptyne-2,5-diamine and 2-methyl-6-heptyne-2,5-diamine, which cannot be substrate of monoamine oxidase, were tested as selective irreversible inhibitors of ornithine decarboxylase. Our results demonstrate that (2R,5R)-6-heptyne-2,5-diamine is greater than 10 times more potent, both in vitro and in vivo, than α-difluoromethylornithine, the most widely used irreversible inhibitor of this enzyme.

INTRODUCTION: Considerable knowledge has accumulated during the last few years on the functional roles of the polyamines putrescine, spermidine and spermine in processes related to cell growth and proliferation (1,2). This progress was mainly achieved by studying the effects of inhibitors of polyamine biosynthesis in prokaryotes and eukaryotes (3,4). Among specific inhibitors of ornithine decarboxylase (EC 4.1.1.17,ODC), the enzyme responsible for the biosynthesis of putrescine, α-difluoromethylornithine (DFMO) has attracted much attention (7,8). Another potent enzyme-activated irreversible inhibitor of mammalian ODC is (R)-5-hexyne-1,4-diamine (7,9). Unfortunately this compound, which inhibits 4-aminobutyrate aminotransferase (EC 2.6.1.19, GABA-T) in vivo, increases brain 4-aminobutyric acid levels and thus, causes secondary pharmacological effects (10). This was explained by the transformation of 5-hexyne-1,4-diamine to 4-aminohex-5-ynoic acid through an oxidative pathway, the first step being catalysed by mitochondrial monoamine oxidase (EC 1.4.3.4, MAO) (10,11). Therefore we decided to modify the molecule to avoid its oxidation by MAO.

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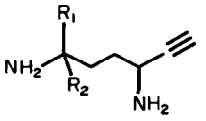

$R_1 = H, R_2 = H$: 5-hexyne-1,4-diamine
$R_1 = CH_3, R_2 = H$: 6-heptyne-2,5-diamine
$R_1 = CH_3, R_2 = CH_3$: 2-methyl-6-heptyne-2,5-diamir

Scheme I

It is known that α-branched primary amines are not substrates of MAO (12). Consequently we have designed 6-heptyne-2,5-diamine and 2-methyl-6-heptyne-2,5-diamine (see scheme I) as new potential selective inhibitors of ODC *in vitro* and *in vivo*. Synthesis of these compounds, resolution, and characterization of the active stereoisomer of 6-heptyne-2,5-diamine will be published elsewhere. We disclose here that (2R,5R)-6-heptyne-2,5-diamine is an extremely potent enzyme-activated irreversible inhibitor of ODC *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals: DL- 1-[^{14}C] ornithine (58 Ci/mole) and S-adenosyl-L-[carboxyl-^{14}C] methionine (61 Ci/mole) were purchased from the Radiochemical Centre, Amersham U.K. All other chemical products were of the purest grade commercially available.

Animals: Male rats of the Sprague-Dawley strain (200g-220g body wt.) were purchased from Charles River, France. Animals had access to standard diet and water *ad libitum* and were kept under a constant 12 h light/12 h dark lighting schedule. They were killed by decapitation at about the same time of day to minimize effects due to diurnal fluctuations. Drugs dissolved in 0.9 % saline were given by gavage. Rats given water served as control.

Assays of enzyme activities: ODC preparation was obtained from the liver of rats which had been injected with thioacetamide (150 mg/kg of body wt.) 18 h before sacrifice, and was purified about 10-fold by acid treatment at pH 4.6 as described by Ono et al. (13). The specific activity of this preparation was 0.2 nmoles of CO_2/min/mg of protein. The kinetic constants of the time-dependent inhibition were determined essentially as described previously (7). S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50, SAM-DC) was prepared and assayed for determination of activation constants of diamines as described previously (14). Preparation of MAO and assay of its activity were likewise performed according to ref. 14. For *in vivo* experiments, ODC, SAM-DC and GABA-T activities were determined according to published methods (14,15).

RESULTS AND DISCUSSION

In vitro studies: Incubation of ODC preparation with 2-methyl-6-heptyne-2,5-diamine, 6-heptyne-2,5-diamine, or (2R,5R)-6-heptyne-2,5-diamine resulted in a time-dependent loss of enzyme activity which followed pseudo first-order kinetics for approximately two half-lives. Over longer time-periods, the semilogarithmic plots deviated from linearity (Fig. 1). Loss of activity was related to the concentration of inhibitor. By plotting the time of half-inactivation ($t_{\frac{1}{2}}$) as a function of the reciprocal of the inhibitor concentration (1/I) according

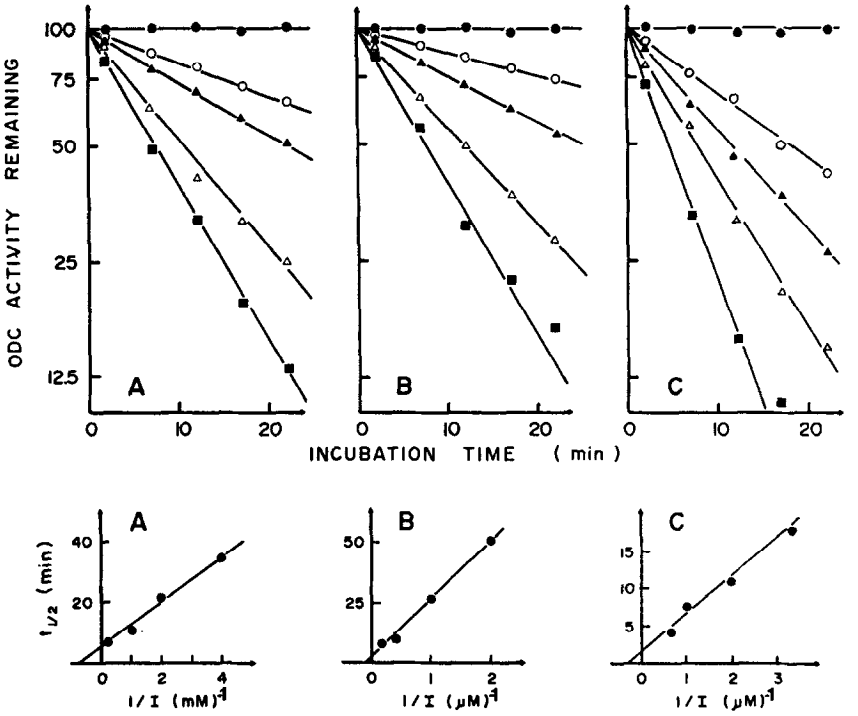

Fig.1 : Time - and concentration - dependent inhibition of rat liver ODC in vitro. ODC was incubated at 37°C with phosphate buffer 30mM pH 7.1, dithiothreitol 5mM, pyridoxal phosphate 0.1 mM and various concentrations of inhibitor : A : 2-methyl-6-heptyne-2,5-diamine (●: control; ○ : 250 μM; ▲ : 500 μM; △ : 1000 μM; ■ : 5000 μM); B : 6-heptyne-2,5-diamine (●: control; ○: 0.5 μM; ▲ : 1 μM; △ : 2.5 μM; ■ : 5 μM); C : (2R,5R)-6-heptyne-2,5-diamine (●: control; ○ : 0.3 μM; ▲ 0,5 μM; △ : 1 μM; ■ : 1.5 μM). In the lower part of the figure, the times of half inactivation ($t_{\frac{1}{2}}$) are plotted against the reciprocal of the inhibitor concentration.

to Kitz and Wilson (16), straight lines were obtained (Fig. 1). These lines did not pass through the origin but intercepted the positive y axis, demonstrating saturation effects which involve the enzyme's active-site in the inhibitory process. Kinetic constants for the time-dependent inhibition of ODC can be extrapolated from Fig.1. The apparent dissociation constants (K_I) for 2-methyl-6-heptyne-2,5-diamine, 6-heptyne-2,5-diamine and (2R,5R)-6-heptyne-2,5-diamine are 1300, 13 and 3 μM, respectively and the times of half-inactivation at infinite concentration of inhibitor ($t_{\frac{1}{2}}$) are 5.4,1.8 and 1.7 min.

Further studies on ODC inactivation by (2R,5R)-6-heptyne-2,5-diamine showed protective effects of the natural substrate S-ornithine and of the competitive inhibitor S-2-methylornithine (17) whereas R-2-methylornithine, which is practically devoid of affinity toward ODC, had no protective effect (Fig.2). These re-

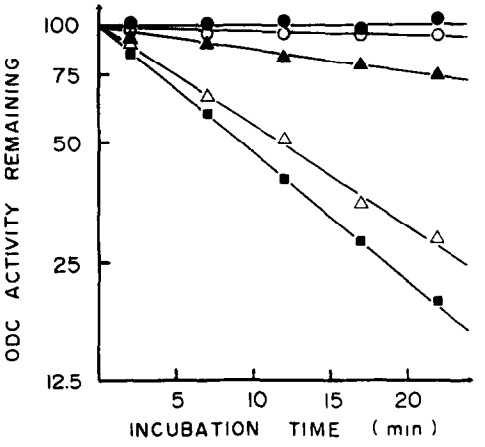

Fig.2 : Protection against the inactivation of rat liver ODC by (2R,5R)-6-heptyne-2,5-diamine (1 μM, ■) : the effect of S-ornithine (1 mM, ▲), S-2-methylornithine (1mM, ○) and R-2-methylornithine (1 mM, △).

sults confirm that the inactivation is indeed active-site directed. Furthermore, the presence of dithiothreitol (5 mM) in the preincubation medium and the absence of a lag-time before the onset of inhibition rule out the possibility of inactivation *via* affinity labeling by a diffusible alkylating species (18). Incubation with (2R,5R)-6-heptyne-2,5-diamine at 10 μM concentration resulted in 98 % inactivation of ODC after 30 min. Prolonged (24 h) dialysis of this inactivated ODC against a buffer solution containing sodium phosphate (30 mM, pH 7.1), PLP (0.1 mM) and dithiothreitol (5 mM) (conditions where the native enzyme is stable) led to partial regeneration of enzyme activity, from 2 % of control before dialysis to 24 % of control after dialysis. Similar recoveries have been observed after dialysis of ODC and aromatic amino acid decarboxylase (EC 4.1.1.26) previously incubated and inhibited by α-ethynyl ornithine and α-ethynyl DOPA respectively (19-21). This does not rule out a covalent linkage of the inhibitor to the enzyme active-site but suggests that this bond, if it exists, can be slowly hydrolyzed (22).

In addition, we found that (2R,5R)-6-heptyne-2,5-diamine, similarly to other diamines (14) does activate SAM-DC but has a weak activation constant (K_a=300 μM) comparatively to putrescine (K_a=16 μM). We also confirmed that, as expected, this compound is not a substrate for MAO.

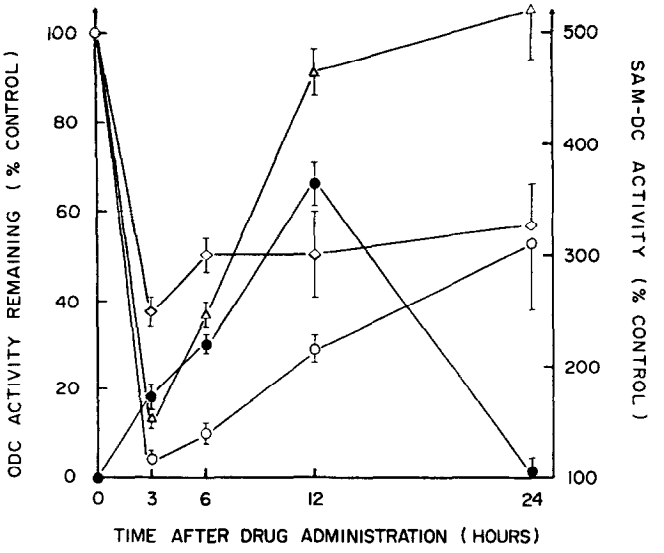

Fig.3 : Effect of a single dose of (2R,5R)-6-heptyne-2,5-diamine on ODC activity in ventral prostate, thymus and testis and on SAM-DC activity in ventral prostate. Rats were given (2R,5R)-6-heptyne-2,5-diamine (25 mg/kg) by gavage at time zero. At given intervals, animals were killed and enzyme activities were immediately measured (○, ODC act. and ●, SAM-DC act. in the ventral prostate; △, ODC act. in thymus; ◇, ODC act. in testis).

In vivo studies: As shown in Fig. 3, maximum inhibition of prostatic ODC occurred 3 to 6 h after a single oral administration of 25 mg/kg (2R,5R)-6-heptyne-2,5-diamine. At these times the remaining ODC activity was 5-10 % of the control values in this organ. Thereafter, ODC activity in the prostate increased slowly to reach 50 % of the control value 24 h after administration. An inverse pattern was observed for SAM-DC activity which was increased four-fold over control values within 12 h and then after returned to control values 24 h after administration of the compound. Similar results concerning increase of SAM-DC activity in the prostate were already described after injection of DFMO. They were explained by a decrease of spermidine concentration in this organ (15,23). The time course of ODC activity in thymus and testis was similar to that observed in prostate, but at all times the decrease of ODC activity was significantly greater in ventral prostate. Fig. 4 shows that the decrease of ODC activity was dose-dependent in the prostate and that (2R,5R)-6-heptyne-2,5-diamine was approximately 10 times more potent than DFMO administered to rats under the same conditions. Similar results were obtained in thymus and testis (not shown). Further

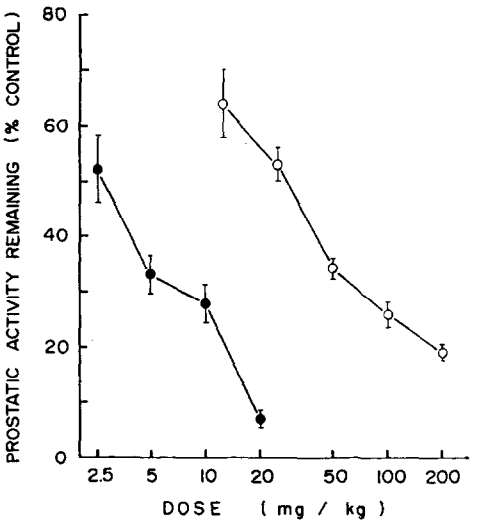

Fig.4 : Comparison of the effects of a single oral dose of (2R,5R)-6-heptyne-2,5-diamine (●) and of DFMO (○) on ODC activity in ventral prostate. ODC activity was measured 6 hours after administration of the compounds to rats.

studies have shown that chronic administration of (2R,5R)-6-heptyne-2,5-diamine given to rats in their drinking water (1.0 g/L) for 8 days produce the same decrease of prostatic polyamines and blockade of growth of ventral prostate was reported for DFMO given at 20 g/L (15). Moreover, as expected, this compound did not produce any inhibition of GABA-T activity in brain.

In conclusion, (2R,5R)-6-heptyne-2,5-diamine is an extremely potent and selective enzyme-activated irreversible inhibitor of ODC at least 10-times more potent than DFMO both in in vitro and in vivo. This compound has of course a high potential as antiproliferative agent not only for experimental use but also for clinical trials of its anti-cancer activity.

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