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BRIEF COMMUNICATION

# α-Difluoromethylornithine Does Not Antagonize the Behavioral Effects of Putrescine

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FERCHMIN, P. A., E. RIVERA AND V. A. ETEROVIĆ. *α-Difluoromethylornithine does not antagonize the behavioral effects of putrescine.* PHARMACOL BIOCHEM BEHAV **45**(4) 967–971, 1993.—α-Difluoromethylornithine (DFMO), a specific inhibitor of putrescine synthesis, is widely used in studies of polyamine function as well as clinically. We studied the effect of DFMO on the tendency to explore in the Greek cross maze that provides the rat with the choice to enter either white or black compartments. After a single injection of 400 mg/kg DFMO, the entries into white compartments were significantly decreased. A similar decrease had been observed previously with 200 mg/kg putrescine. Simultaneous administration of DFMO (400 mg/kg) and putrescine (200 mg/kg) resulted in decreased entries into both white and black compartments. When 400 mg/kg DFMO plus 400 mg/kg putrescine were injected, the entries into both compartments were further decreased and the time spent in white compartments was also decreased. This pattern mimicked that found with anxiogenic drugs. Injection of DFMO (400 mg/kg) produced no change in either putrescine, spermidine, or spermine concentration measured in brain cortex. Putrescine (200 mg/kg) plus DFMO produced the same transient increase in cortical putrescine as putrescine alone. We conclude that DFMO is mildly anxiogenic and that this activity is independent of its inhibition of putrescine synthesis.

α-Difluoromethylornithine  Putrescine  Anxiogenic  Brain cortical polyamines  White-dark maze  Greek cross maze

α-DIFLUOROMETHYLORNITHINE (DFMO) is a specific and extensively used inhibitor of ornithine decarboxylase (ODC) (15). ODC is the rate-limiting enzyme in the synthesis of putrescine, the smallest of the natural polyamines and the precursor of spermidine and spermine. Due to the regulatory importance of the ODC/polyamine system, DFMO is being used in numerous brain studies in vivo and in vitro (5,7, 9,11,18). In addition, because of its antiproliferative, antitrypanosomal, and neuroprotective properties, DFMO is used in preclinical and clinical studies and in clinical practice (9,16). It is, therefore, important to determine the behavioral effects of DFMO.

In the present work we analyzed the effect of DFMO on rat behavior in the Greek cross maze. This maze provides the subject with the choice of exploring white or black compartments. In that respect, the Greek cross maze is similar to the Crawley and Goodwin test which, in its original form or with modification, is routinely used to assess the effect of drugs on anxiety (2,14).

We report here that DFMO decreased the number of entries into white compartments as if it were mildly anxiogenic. This effect was increased, rather than inhibited, by putrescine. This apparent paradox suggests that this behavioral effect of DFMO is not mediated by ODC inhibition.

## METHOD

### *Subjects*

One hundred forty-four Sprague-Dawley albino male rats bred in our colony were used for behavioral testing: fifty-six for each Experiment I and II, and 32 for Experiment III. Forty-eight additional rats of the same strain, sex, and age

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were used for the determination of cortical polyamines in Experiments IV and V. Within 4 days after birth each litter was reduced to eight pups, leaving the maximum number of males. During the preweaning period the animals were habituated to handling. The animals were weaned at 30 days of age and kept in standard laboratory conditions until the beginning of the experiment, 1 to 6 days later.

*Behavioral Test and Pharmacological Treatments*

Testing in the Greek cross maze was as described previously (6). Briefly, the Greek cross maze is a cross-shaped structure with a central gray compartment communicated through 6 cm semicircular holes with four peripheral compartments. Two of these, on opposite sites of the central compartment, are painted black, while the other two are white. The walls of the maze were 38 cm high and all five compartments measured 21 × 21 cm. The test was started by putting the rat in the middle of the gray compartment. During the next 5 min the time of entries into the compartments was manually recorded by two independent observers. Entries were scored when a rat introduced at least the head and the front paws into a compartment. The values obtained by the two observers differed by less than 10% in 98% of the cases for measurements of the number of entries and in 92% of the cases for measurements of the time spent in each compartment.

α-Difluoromethylornithine hydrochloride was a gift of Marion Dow Merrell Company, putrescine dihydrochloride was purchased from Sigma Chemical Co. and *N*-methyl-β-carboline-3-carboxamide (β-CCA) from Research Biochemicals Inc. DFMO and putrescine were injected intraperitoneally as isotonic neutral solutions and β-CCA as slightly acid isotonic saline solution.

*Dissection of Cerebral Cortex and Determination of Polyamines*

The procedure for the dissection of the cerebral cortex was described in (6). After dissection, the cerebral cortex was weighed, frozen in liquid nitrogen, and stored at −80°C. Polyamines were determined as described in (7). Briefly, the frozen tissue samples were homogenized in 5% trichloroacetic acid, neutralized with an excess of NaOH, and derivatized with benzoylchloride. The benzoylated polyamines were separated isocratically with 40% acetonitrile and 60% water on a 15 cm long C18 Ultrasphere Altex column using the QA1 HPLC apparatus from Waters.

*Experimental Design and Statistical Analysis*

Each experiment consisted of several blocks of four pairs of littermates. Within each pair, the rats were assigned at random to saline or drug condition. Within each block, the pairs were assigned at random to the four testing times: 15 min, 2, 4, or 6 h after injection of the drug or saline solution. Thus a randomized block design was superimposed on a factorial 2 × 2 design. One main factor was the presence of the drug and the other the time elapsed between injection and testing. All behavioral and biochemical data were normally distributed and homoscedastic. They were analyzed by two-way analysis of variance for blocked data.

## RESULTS

*Experiment I: Effect of DFMO on Behavior in the Greek Cross Maze*

Rats were tested in the Greek cross apparatus either 15 min, 2, 4, or 6 h after injection of 400 mg/kg of DFMO or saline solution. Two replicates of this experiment were done; the first (Experiment Ia) with 24 rats (12 DFMO/saline pairs, three at each time point) and the second (Experiment Ib) with 32 animals (16 pairs, four at each time point).

The statistical analysis of entries into white or black compartments revealed no significant effects for time after injection or for interaction between time and drug. Therefore, the data from observations done at different times after injection were pooled and only the pooled results are presented in Table 1.

Treatment with DFMO significantly reduced the entries into white compartments [−19%, $F(1, 48) = 4.9$, $p < 0.05$]. The entries into black compartments and the time spent in each type of compartment were not significantly affected.

*Experiment II: The Behavioral Effects of DFMO Were Not Reversed by Simultaneous Administration of Putrescine*

If DFMO affected behavior through a decrease in brain putrescine level, simultaneous administration of DFMO and putrescine should result in attenuation of behavioral effects. This hypothesis was tested in Experiment II where a mixture of 400 mg/kg of DFMO and 200 mg/kg of putrescine was injected into rats previous to the Greek cross test. Two replicates of this experiment were done: Experiment IIa, with 12 pairs of animals and Experiment IIb with 16 pairs. The experimental design was as described for Experiment I. The results were not affected by the time after injection and so only the pooled data are presented in Table 2.

The simultaneous injection of DFMO and 200 mg/kg of putrescine significantly decreased entries into both white [−19%, $F(1, 48) = 5.0$, $p < 0.05$] and black [−22%, $F(1,$

TABLE 1

EFFECT OF DFMO ON THE TENDENCY TO EXPLORE IN THE GREEK CROSS MAZE

| | Saline Mean ± SEM | DFMO Mean ± SEM | Percent Difference |
|---|---|---|---|
| Entries to white compartments | | | |
| Experiment Ia | 7.5 ± 0.7 | 5.9 ± 0.8 | −21 |
| Experiment Ib | 6.8 ± 0.4 | 5.5 ± 0.5 | −19 |
| Total | 7.0 ± 0.4 | 5.7 ± 0.4 | −19* |
| Entries to black compartments | | | |
| Experiment Ia | 8.4 ± 0.8 | 6.8 ± 0.9 | −19 |
| Experiment Ib | 7.7 ± 0.7 | 7.9 ± 0.8 | 3 |
| Total | 8.0 ± 0.5 | 7.4 ± 0.6 | −8 |
| Time spent in white compartments (s) | | | |
| Experiment Ia | 85.1 ± 10.5 | 102.8 ± 23.7 | 21 |
| Experiment Ib | 80.6 ± 7.6 | 70.7 ± 8.4 | −12 |
| Total | 82.5 ± 6.1 | 84.4 ± 11.4 | 2 |
| Time spent in black compartments (s) | | | |
| Experiment Ia | 83.0 ± 12.4 | 81.5 ± 16.4 | −2 |
| Experiment Ib | 104.2 ± 9.2 | 128.9 ± 12.7 | 24 |
| Total | 95.1 ± 7.6 | 108.6 ± 10.9 | 14 |
| Total number of entries | | | |
| Experiment Ia | 15.9 ± 0.6 | 12.7 ± 1.2 | −20* |
| Experiment Ib | 14.6 ± 0.8 | 13.4 ± 1.2 | −8 |
| Total | 15.1 ± 0.5 | 13.1 ± 0.8 | −13* |

The dose of DFMO was 400 mg/kg. The numbers of rats used were 12 pairs in Experiment Ia and 16 pairs in Experiment Ib.

*$p < 0.05$.

TABLE 2

EFFECT OF SIMULTANEOUS ADMINISTRATION OF PUTRESCINE (200 mg/kg) AND DFMO (400 mg/kg) ON THE TENDENCY TO EXPLORE IN THE GREEK CROSS MAZE

| | Saline Mean ± SEM | Putrescine plus DFMO Mean ± SEM | Percent Difference |
|---|---|---|---|
| Entries to white compartments | | | |
| Experiment IIa | 5.2 ± 0.6 | 4.6 ± 0.3 | −12 |
| Experiment IIb | 6.1 ± 0.5 | 4.6 ± 0.6 | −25* |
| Total | 5.7 ± 0.4 | 4.6 ± 0.3 | −19* |
| Entries to black compartments | | | |
| Experiment IIa | 11.0 ± 0.5 | 8.6 ± 0.6 | −22† |
| Experiment IIb | 10.1 ± 0.7 | 7.8 ± 0.7 | −23* |
| Total | 10.5 ± 0.5 | 8.1 ± 0.5 | −23† |
| Time spent in white compartments (s) | | | |
| Experiment IIa | 43.0 ± 5.7 | 62.0 ± 14.8 | 44 |
| Experiment IIb | 55.7 ± 4.6 | 68.6 ± 17.0 | 23 |
| Total | 50.3 ± 3.7 | 65.8 ± 11.4 | 31 |
| Time spent in black compartments (s) | | | |
| Experiment IIa | 158.7 ± 9.9 | 152.2 ± 16.0 | −4 |
| Experiment IIb | 138.8 ± 7.3 | 129.7 ± 15.4 | −7 |
| Total | 147.3 ± 6.2 | 139.3 ± 11.2 | −5 |
| Total number of entries | | | |
| Experiment IIa | 16.2 ± 0.7 | 13.2 ± 0.7 | −19* |
| Experiment IIb | 16.2 ± 1.1 | 12.4 ± 1.2 | −24* |
| Total | 16.2 ± 0.7 | 12.7 ± 0.8 | −22‡ |

The numbers of rats used were 12 pairs in Experiment IIa and 16 pairs in Experiment IIb.
*$p < 0.05$.
†$p < 0.005$.
‡$p < 0.001$.

48) = 14.4, $p < 0.005$] compartments. The time spent in both types of compartments did not differ from controls injected with saline solution.

This pattern of changes is inconsistent with the notion that the effects of DFMO are mediated by a decrease in putrescine concentration.

*Experiment III: A Higher Dose of Putrescine, Still Did Not Reverse the Behavioral Effects of DFMO*

In Experiment III (Table 3) a higher dose of putrescine (400 mg/kg) was administered simultaneously with 400 mg/kg of DFMO. Sixteen pairs of rats were used. The experimental design was as in Experiment I.

This treatment caused a larger than 50% decrease in white [−57%, $F(1, 24) = 29.7$, $p < 0.001$], black [−55%, $F(1, 24) = 101.8$, $p < 0.0001$] and total entries [−56%, $F(1, 24) = 46.0$, $p < 0.0001$]. The time spent in white compartments was decreased significantly [−47%, $F(1, 22) = 23.9$, $p < 0.0001$], but the time spent in black compartments did not change.

These results strengthened the conclusion that DFMO was affecting behavior by a mechanism different from a decrease in putrescine concentration.

*Experiment IV: Effect of DFMO on the Levels of Cortical Polyamines*

This experiment was performed to measure the effect of DFMO on cortical polyamine concentration under the experimental conditions used in the behavioral studies.

Twelve pairs of rats were used; 400 mg/kg of DFMO was injected into one member of each pair, an equal volume of saline solution into the other. The animals were sacrificed at 15 min, 2, 4, or 6 h after injection, three pairs at each time point. Brain cortices were dissected and polyamine concentration determined as explained in the Method section.

The concentration of putrescine, spermidine, and spermine in cerebral cortex did not change after a single injection of 400 mg/kg DFMO (see Fig. 1A for putrescine; data for spermidine and spermine are not shown).

*Experiment V: How the Mixture of DFMO and Putrescine Affected Cortical Polyamine Concentration*

In an experiment designed similarly to Experiment IV, cortical polyamine concentration was measured after injection of a mixture of 200 mg/kg putrescine and 400 mg/kg DFMO. A total of twelve pairs of animals were used, three at each time point (15 min, 2, 4, and 6 h after injection).

This treatment caused a sharp increase in the level of cortical putrescine that decreased rapidly with time (Fig. 1B). This pattern did not differ from that observed after injection of putrescine alone (dotted line on Fig. 1B). The levels of spermidine and spermine were not changed.

Therefore, under our experimental conditions, DFMO affected neither total putrescine concentration in cerebral cortex nor its decay after administration of exogenous putrescine.

## DISCUSSION

The behavioral test used here, the Greek cross maze, measures two opposing tendencies of the rat: the tendency to explore a novel environment and the fear of a bright place. The emotional state of the animal determines the balance between these two predispositions. In other black and white tests, anxiogenic drugs decrease the entries into white compartments while anxiolytic drugs have the opposite effect (10,14). Preliminary results from our laboratory indicate that the Greek cross maze is no exception: a moderate dose (20 mg/kg) of the anxiogenic drug *N*-methyl-β-carboline-3-carboxamide (β-CCA) decreased the entries into white compartments by 62%,

TABLE 3

EFFECT OF PUTRESCINE (400 mg/kg) PLUS DFMO (400 mg/kg) ON THE TENDENCY TO EXPLORE IN THE GREEK CROSS MAZE

| | Saline Mean ± SEM | Putrescine plus DFMO Mean ± SEM | Percent Difference |
|---|---|---|---|
| Entries to white compartments | 7.0 ± 0.7 | 3.0 ± 0.4 | −57* |
| Entries to black compartments | 9.4 ± 0.4 | 4.3 ± 0.5 | −54† |
| Time spent in white compartments (s) | 67.5 ± 5.4 | 35.4 ± 6.3 | −48† |
| Time spent in black compartments (s) | 133.6 ± 9.1 | 153.3 ± 20.6 | 15 |
| Total number of entries | 16.5 ± 0.8 | 7.2 ± 0.9 | −56† |

Sixteen pairs of rats were used.
*$p < 0.001$.
†$p < 0.0001$.

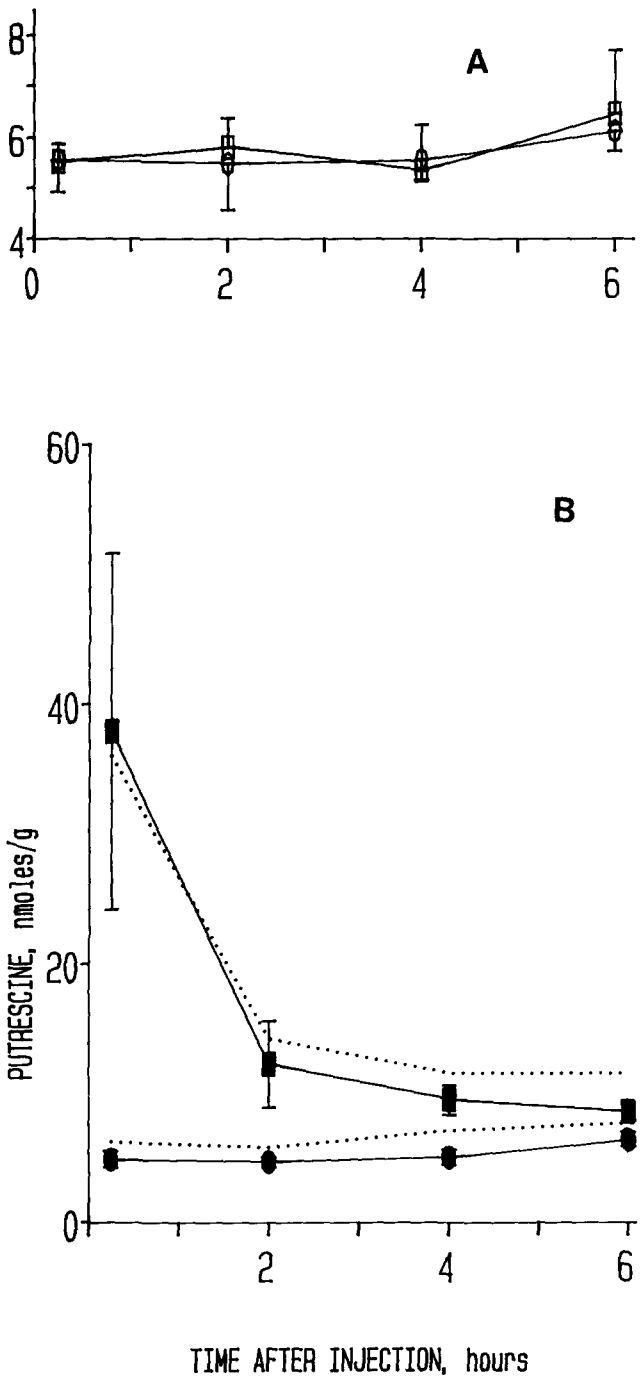


FIG. 1. (A) Putrescine levels in brain cortex after injection of DFMO. The concentration of putrescine is shown 15 min, 2, 4, or 6 h after a single injection of 400 mg/kg of DFMO (squares) or saline solution (circles). A total of 24 rats were used; thus each point is the average of the values for three cortices. Vertical bars are standard errors; where not visible they are smaller than the size of the symbol. Saline and DFMO groups did not significantly differ. (B) Putrescine levels in brain cortex after simultaneous administration of 400 mg/kg DFMO and 200 mg/kg putrescine. The experiment was as described above. The two groups significantly differed for the main factor 'drug,' $F(1, 16) = 11.1$, $p < 0.004$, for the main factor 'time,' $F(1, 16) = 3.72$, $p < 0.03$, and for the interaction between these two factors, $F(1, 16) = 4.1$, $p < 0.025$. The dotted line represents the results from a similar experiment in which 200 mg/kg putrescine without DFMO was injected in a previous work (6).

while the entries into black compartments decreased by only 32% ($n = 4$). A higher dose of β-CCA (40 mg/kg) decreased the entries into both white (−91%) and black (−80%) compartments and the time spent in the white compartments (−79%); $n = 8$, $p < 0.05$ by paired $t$-test for all three measurements. The time spent in black compartments did not differ from saline controls.

By these criteria, DFMO acted as a mildly anxiogenic drug, lowering specifically the entries into white compartments. This conclusion is strengthened by the fact that DFMO does not affect general locomotor activity (1).

Why did DFMO, a potent ODC inhibitor, fail to lower brain putrescine concentration? The answer to this question is based on the strict regulation of brain polyamine levels. In older rats, as opposed to neonates (19), ODC inhibition is compensated by a decrease in polyamine turnover, increase in ODC synthesis, and several other mechanisms (1,3,17). That DFMO is actually inhibiting ODC in brains of 35-day-old rats is indicated by the fact that putrescine concentration is decreased by 50% after three daily injections (7,17).

The lack of a change in putrescine concentration suggested that the behavioral effects of DFMO observed here are not mediated by ODC inhibition. The same conclusion follows from the fact that simultaneous administration of putrescine did not attenuate DFMO effects. Furthermore, we have recently reported that putrescine per se also decreased the tendency to explore in the Greek cross maze (6). While a low dose (200 mg/kg) inhibited preferentially the entries into white compartments, a higher dose (400 mg/kg) depressed similarly the entries into both black and white compartments. Cyclohexylamine, a drug that increases endogenous putrescine concentration, produced effects similar to a high dose of putrescine and to the higher dose of β-CCA. Thus both these substances produced results similar to those of DFMO, not opposite.

We have recently demonstrated that DFMO inhibits GABAergic transmission in hippocampal slices independently of a decrease in putrescine levels (8). Thus DFMO putative anxiogenic effects could be mediated by the GABAergic system, whose relation to anxiety is well established.

Biological effects of DFMO unrelated to ODC inhibition have been observed before. De Sarro et al. (4) reported that DFMO produced electroencephalographic changes and potentiated the convulsant properties of putrescine, probably by interference with GABAergic transmission. Electroencephalographic changes in patients infected with Trypanosoma gambiense and treated with DFMO suggested that DFMO had convulsant activity (9,16). Therefore, although DFMO is a very specific ODC inhibitor, it is not without additional effects (20).

It is important to recognize these additional activities of DFMO because of the widespread use of this drug in experimental and preclinical studies. The convulsant (4) and anti-GABAergic (8) effects of DFMO could be an important problem with its proposed use as a neuroprotective drug (11–13). If the anxiogenic activity described here also expressed itself in humans, this could cause serious clinical problems with the use of DFMO as an antineoplastic and antiparasitic drug (16).

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