Protective Effect of 2-Diethylaminoethyl 4-Methylpiperazine-1-Carboxylate in Mice Infected with Influenza Virus. (30699)

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In the course of screening compounds for antiviral activity, 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate was found to have a protective effect in mice infected with influenza virus. The compound was evaluated, compared with 1-adamantanamine(1), and tested in combination with this substance. Results of these tests are reported here.

Materials and methods. 2-Diethylaminoethyl 4-methylpiperazine-1-carboxylate is a colorless oil and 1-adamantanamine·HCl is a colorless crystalline salt. The compounds* are readily soluble in water and were administered to the mice by oral tubing.

The following strains of influenza virus were used: type A, PR-8; type A1, Ann Arbor 1/57; type A2, Japan 305/57; type B, Lee; type B, Great Lakes 1739. The stock virus in each case consisted of the supernatant of a 10% homogenate in Brain Heart Infusion broth (Difco) of pooled lungs from infected mice.

Male white mice (Taconic Farms) of 18 to 22 g body weight were used. They were infected, while under slight ether anesthesia, by intranasal instillation of 0.05 ml of appropriate broth dilutions of stock virus.

The acute lethal toxicity of the piperazinecarboxylate for mice was determined in nonfasted, noninfected animals. The LD_{50} and the 95% confidence limits were obtained by the probit method.

Results. The acute oral LD_{50} of the piperazinecarboxylate was 2500 mg/kg with 95% confidence limits of 2200 to 2900 mg/kg.

Table I shows the effect of graded oral doses of the compound in mice infected with dilutions of influenza A PR-8 strain virus which killed 96% of nontreated mice. A dose of 1600 mg/kg protected 82% of the mice.

TABLE I. Effect of 2-diethylaminoethyl, 4-methylpiperazine-1-carboxylate on Survival of Mice Infected with Influenza A, PR-8 Strain Virus.

Single oral dose† (mg/kg)	Survivors on 14th day after infection: Per 20 mice in each of 5 successive tests	Per 100 mice (pooled data)	Mean survival time in days‡
1600	16, 17, 16, 17, 16	82*	8§
800	16, 14, 11, 17, 16	74*	10
400	5, 12, 8, 10, 7	42*	8
200	1, 4, 1, 1, 4	11	8
Controls	1, 0, 0, 3, 0	4	8

* Statistically different from controls at 1% level of significance.
† Administered immediately after infection.
‡ Mean survival time of mice that died.
§ Five mice died within 4 days after infection presumably due to drug toxicity.

This dose may have had some toxic effects since a few of the mice died before the nontreated control mice began to die from infection. The 800 mg/kg dose was considered to be the maximum tolerated dose; it protected 74% of the mice. A dose of 400 mg/kg protected 42% of the mice.

The effect of multiple doses, and of varying the start of treatment, was examined. Multiple doses were no more effective than a single dose given at the time of treatment. The effectiveness of the piperazinecarbozylate was critically dependent on time of administration in relation to time of infection. Treatment started one hour prior to infection was less effective than treatment started at time of infection. Treatment delayed until one hour after infection was not effective.

The effectiveness of the compound was tested in mice infected with graded infective doses of various influenza viruses. The results (Fig. 1) showed that the piperazinecarboxylate given orally at a dose of 800 mg/kg protected a significant number of mice infected with the PR-8, the Ann Arbor and the Japan 305/57 strains. It can be seen that the compound was more effective against the

* The compounds were synthesized by Dr. R. B. Angier, Dr. K. C. Murdock and Mr. W. V. Curran of the Organic Chemical Research Section of these Laboratories.

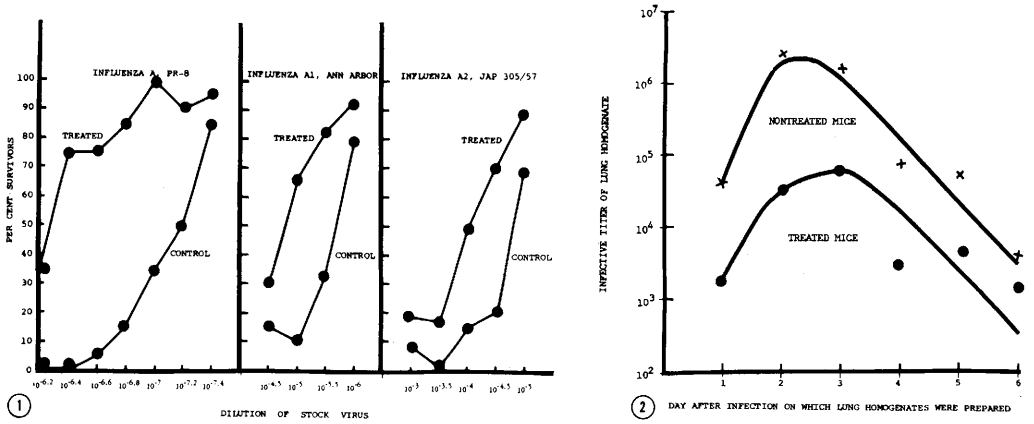

FIG. 1. Effect of treatment with 2-diethylaminoethyl, 4-methylpiperazine-1-carboxylate on survival of mice infected with graded doses of various influenza viruses. Twenty control and 20 treated mice were used per each dilution of each stock virus. Survival on 14th day is shown. Treated mice received a dose of 800 mg/kg of piperazinecarboxylate by oral tubing within 10 minutes after infection; control mice received 1 ml of 0.85% NaCl solution.

FIG. 2. Effect of treatment with 2-diethylaminoethyl, 4-methylpiperazine-1-carboxylate on the virus concentration in the lungs of mice infected with influenza A, PR-8 virus. One hundred and twenty mice were infected with an LD_{95} of virus. One group of sixty mice was treated with a single dose of the piperazinecarboxylate (800 mg/kg, by oral tubing, within 10 minutes after infection), the other group of sixty mice was used as control. Each day thereafter, 10 mice in each group were killed and homogenates of pooled lungs were prepared. The infectivity of each homogenate was titrated in mice (10 mice per dilution). In this test each mouse surviving for the entire 14-day test period was assigned a survival time of 14 days. The infective titer is the reciprocal of the dilution producing an average survival time of 8 days.

PR-8 strain than against the two other strains. The piperazinecarboxylate was inactive against infections in mice produced by the Columbia SK virus, the MEF-1 strain of poliomyelitis virus, Herpes simplex virus and Rabies virus.

In studies on the mode of action, the direct effect of the compound on virus was examined. Suspensions of PR-8 virus incubated for one hour at 37°C in a 0.85% NaCl solution containing 1% of the piperazinecarboxylate and control suspensions without the compound, were titrated in mice. The results showed that both viral suspensions had the same lethality. The compound, thus, did not inactivate the virus on contact *in vitro*.

Treatment of the mice with the piperazinecarboxylate inhibited viral multiplication in the lungs of mice. Fig. 2 shows that the peak concentration of virus was reached on the second day after infection in the control mice and on the third day in the treated mice. Also, the concentration of infective virus in the lungs of treated mice was held down to about one-tenth of the concentration reached in control mice.

Since the piperazinecarboxylate inhibited, but did not completely suppress viral multiplication in the lungs of mice, it would be expected that mice surviving an infection due to treatment with the compound had opportunity to develop immunity. Indeed, mice protected by the piperazinecarboxylate from an infection with PR-8 virus were found to be immune to a subsequent challenge with the same virus.

The piperazinecarbozylate was compared with 1-adamantanamine. 1-Adamantanamine was more effective than the piperazinecarboxylate against the Ann Arbor and the Japan 305/57 strain. Doses of 1-adamantanamine as small as 5 to 15 mg/kg protected a significant number of mice infected with LD_{95} doses of these viruses. The piperazinecarboxylate showed an effect only at doses near the maximum tolerated one. Against the PR-8 virus, the piperazinecarboxylate was more effective than 1-adamantanamine. A comparison of the effectiveness of the two compounds, each given at maximum tolerated dose, is shown in Table II. The piperazinecarboxylate like 1-adamantanamine was ineffective against

TABLE II. Effect of Treatment with 2-diethylaminoethyl, 4-methylpiperazine-1-carboxylate and 1-Adamantanamine HCl on Survival of Mice Infected with Various Strains of Influenza Virus.

Drug	Single oral dose* (mg/kg)	Survivors per 20 mice on 14th day after infection with: Influenza A (PR-8)	Influenza A1 (Ann Arbor)	Influenza A2 (Jap 305)
Piperazinecarboxylate	800	11†	15†	6
1-Adamantanamine	200	5	18†	16†
Controls	—	1	2	0

* Drugs were administered immediately after infection.
† Statistically different from controls at 1% level.

infections produced by the type B Lee or Great Lakes strains.

The effectiveness of mixtures of the piperazinecarboxylate and 1-adamantanamine against the influenza A (PR-8) infection in mice was examined. The results (Table III) showed that doses of each compound, having little or no effect when given separately, provided significant protection when given as a mixture.

Discussion. The results show that 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate is effective against certain lethal influenza infections in mice. The therapeutic index of the compound was low: protection was achieved only with doses near the maximum tolerated one. These results and other studies† suggest that the compound has little promise of practical value.

To be effective the compound had to be given at the time of infection. The compound did not inactivate the virus *in vitro,* but in mice it inhibited viral multiplication in the lungs. The results suggested that the compound exerted its antiviral effect by interfering with steps in the early phase of infection. Studies on the mode of action of the piperazinecarboxylate on viruses in tissue cultures, indeed, have shown that it acts by blocking penetration of the cells by the virus(2).

TABLE III. Effect of Treatment with 2-diethylaminoethyl, 4-methylpiperazine-1-carboxylate and 1-Adamantanamine HCl Alone and in Combination on the Survival of Mice Infected with Influenza A, PR-8 Strain Virus.

Single oral doses*		Survivors on 14th day after infection:	
Piperazine-carboxylate (mg/kg)	1-Adamantan-amine (mg/kg)	Per 20 mice in each of 3 successive tests	Per 60 mice (pooled data)
400	100	11, 16, 16	43†
400	0	1, 1, 5	7
0	100	4, 6, 5	15
200	50	8, 10, 10	28†
200	0	0, 3, 0	3
0	50	3, 2, 1	6
0	0	0, 2, 0	2

* Drugs alone and in combination were given immediately after infection.
† Statistically different from 1-Adamantanamine or from piperazine-carboxylate alone at 1% level.

Summary. 2-Diethylaminoethyl 4-methylpiperazine-1-carboxylate given orally protected a significant number of mice from the lethal effect of infections with strains of influenza A, A1, and A2. The compound was effective only when given immediately before or after infection. The compound did not inactivate the virus on contact *in vitro,* but in mice it held the virus concentration in the lungs at about one-tenth that reached in controls. Mice protected by the piperazinecarboxylate from an influenza infection were immune to a subsequent challenge with the same virus. The piperazinecarboxylate was more active than 1-adamantanamine against the PR-8 strain of virus, but less active than 1-adamantanamine against the Jap 305/57 strain and the Ann Arbor 1/57 strain. Neither of the 2 compounds was effective against strains of influenza B. Mixtures of the piperazinecarboxylate and of 1-adamantanamine were more effective in the influenza

† Toxicology studies on 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate have been carried out by Dr. J. Noble and Dr. M. Vinocur of these Laboratories. The results showed that the compound was toxic for rats, dogs and monkeys at doses below those showing antiviral activity in mice.

A (PR-8) infection than either of the compounds given alone.

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Effect of 2-Diethylaminoethyl 4-Methylpiperazine-1-Carboxylate on Influenza Viruses in Tissue Culture. (30700)

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Significant protection of mice infected with influenza virus has been demonstrated with 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate(1). This report describes the effect of this compound on various types of influenza virus in tissue culture. In several experiments the compound was compared with 1-adamantanamine(2).

Materials and methods. The influenza viruses were grown in Rhesus monkey kidney monolayers in tubes containing Earle's lactalbumin hydrolysate. The 50% tissue culture infecting dose ($TCID_{50}$) was determined by the hemadsorption test(3). The strains of influenza virus used and their $TCID_{50}$ were as follows: Type A, PR-8 ($10^{7.7}$/ml); type A1, Ann Arbor 1/57 ($10^{6.2}$/ml); type A2, Japan 305/57 ($10^{5.7}$/ml); type B, Lee ($10^{4.9}$/ml); type B, Great Lakes ($10^{5.2}$/ml).

2-Diethylaminoethyl 4-methylpiperazine-1 carboxylate and 1-adamantanamine hydrochloride* were dissolved in Earle's lactalbumin hydrolysate and added to the tissue cultures in 0.1 ml volumes. The tissue culture systems except where indicated contained a final volume of 2 ml (pH 7.2).

The presence of virus or viral growth was determined by the hemagglutination titer and the infectivity titer. At the end of the incubation period, the tissue cultures were subjected to 2 cycles of freezing in the acetone-dry ice bath and thawing at room temperature to release intracellular virus. The hemagglutination titer of the supernatant fluid was then determined by the Salk procedure modified to use the Microtiter system(4) (Cooke Engineering Co., Alexandria, Va.). The concentration of infective virus was determined by titration in mice. Male white mice (Taconic Farms) weighing 18 to 22 g each were infected, while under slight ether anesthesia, by the intranasal instillation of 0.05 ml volumes of appropriate dilutions of the supernatant fluid of the frozen and thawed tissue cultures.

Antiserum against influenza A PR-8 strain virus was obtained from mice 21 days after infection with PR-8 virus. A 1:5 dilution of the serum in lactalbumin hydrolysate was used as stock.

The effect of the compounds on the adsorption of the virus to the cells was examined as follows: The virus inoculum was added to monkey kidney monolayers with and without the compounds and the hemagglutination titer of the supernatant fluid determined at intervals thereafter. The test was carried out at 5°C because at this temperature adsorption of the virus to the cells occurs, but not the subsequent steps of the infection process(5).

The effect of the piperazinecarboxylate on adsorption of the virus to the cells was also studied by exposing the virus inoculum in tissue cultures at 37°C to the compound for various periods. At the end of each exposure

* The compounds were synthesized by Dr. R. B. Angier, Dr. K. C. Murdock and Mr. W. V. Curran, Organic Chemical Research Section, Lederle Laboratories.