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INHIBITORY ACTIVITY OF 2-AMINOOXAZOLE DERIVATIVES AGAINST COXSACKIE B1 VIRUS IN FL CELLS

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Summary. — The antiviral activity of three 2-aminooxazoles was studied against coxsackie B1 virus in FL cells. The virus-induced cytopathic effect was nearly completely suppressed by two of the test compounds as revealed by spectrophotometric turbidity measurements. The plaque reduction amounted to 95—100 per cent. The compounds had no effect on extracellular virus or on adsorption and penetration. The inhibition of infectious virus yield in one-step growth experiments reached 94—99.9 per cent. One of the compounds showed no inhibition of cellular RNA synthesis, whereas viral RNA synthesis was completely suppressed. The other two exerted only a small influence on cellular RNA synthesis. However, the results suggested an inhibition of uridine transport.

Key words: 2-aminooxazoles; antiviral action; cellular and viral RNA synthesis; uridine transport; Coxsackie B1 virus

Introduction

To our knowledge nothing is known about 2-aminooxazoles as antiviral inhibitors. In a screening programme. 3 of 84 newly synthesized 2-aminooxazoles showed an antiviral activity against coxsackie B1 virus in FL cells. This action was confirmed also in other tests. The results of these studies, as well as those on the mode of action of the compounds are described in this paper.

Materials and Methods

Virus and cell cultures. Coxsackie B1 virus (genus *Enterovirus*), strain Ohio, FL cells, media and cultivation conditions were the same as described by Tonew and Tonew (1971).

Test compounds. Z 190/69: 2-guanidino-4,5-di-n- propyl-oxazole-HCl; Z 40/70: 2- guanidino--4,5-diphenyl-oxazole-HCl; and Z 12/73: 2-amino-4,5-diphenyl-oxazole were synthesized and kindly provided by Dr. H. Ulbricht (Ulbricht *et al.*, 1975). The first two compounds were dissolved in distilled water, the last one in methylglycol, and further diluted 1 : 100 and more with maintenance medium.

Test methods. The agar diffusion plaque inhibition test (PDT) and plaque reduction test (PRT) were performed as described by Tonew and Tonew (1969). The influence on virus adsorption

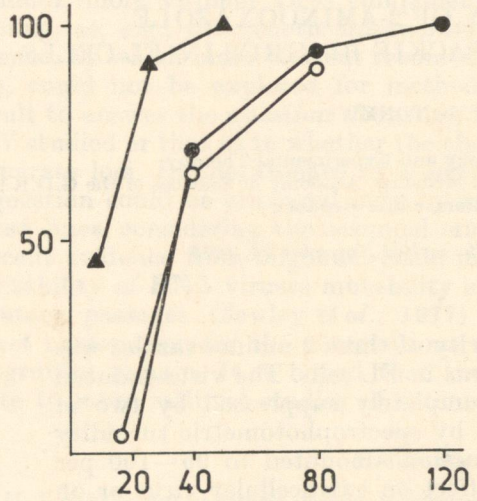

Fig. 1.
Reduction of coxsackie B1 virus plaque numbers in FL-cell monolayers by different concentrations of Z 190/69 (●), Z 40/70 (○) and Z 12/73 (▲) in the agar overlay medium
Abscissa: compound concentration in μM; ordinate: plaque reduction in per cent of untreated control

and penetration processes and on the replication in one-stop growth cycle experiments was studied as described by Tonew and Tonew (1971). The virus-induced cytopathic effect (CPE) was tested according to Tonew *et al.* (1973) and Augsten and Tonew (1975) using an UFD 100 (Vitatron) spectrophotometer.

Infectivity was assayed in tubes by the endpoint method of Reed and Muench and the titres were expressed in $TCID_{50}$ values or by the plaque method in Demeter flasks.

Radiobiological experiments. To investigate RNA synthesis, ^{3}H-uridine was used as precursor. The method of cumulative labelling (simultaneous addition of compounds and precursor) was compared with the prelabelling method (addition of the precursor for 1 hr at 16 °C before application of the test compounds and shift up to 37 °C for incubation) (Tonew and Tonew, 1974). The incorporation was interrupted by adding 0.15% sodium deoxycholate (DOC). After 5 min shaking, the cell suspension obtained was precipitated with 6% trichloroacetic acid, filtered on filter paper disks FN 7 (VEB Niederschlag/Erzgeb., G.D.R.) and washed out in the usual manner. Radioactivity was estimated in the acid-precipitated material in toluene scintillator in a Packard liquid scintillation counter.

Statistical evaluation. The regression straight lines were calculated and the concentrations of the test compound giving the defined inhibition of 1.7 log units (including the 95 per cent confidence intervals) were determined as described by Tonew *et al.* (1974).

Compounds and isotopes. Actinomycin D was obtained from Serva, Heidelberg, F.R.G.; sodium deoxycholate from VEB Berlin-Chemie, Berlin-Adlerhof, G.D.R.; PPO and dimethyl-POPOP from Packard Instruments International, Zürich, Switzerland; and 5-^{3}H-uridine (specific activity 24.4 Ci/mmol) from the Radiochemical Centre, Amersham, England.

Results

Estimation of the antiviral activity

In a concentration of 5 mM, the 2-aminooxazole derivatives showed moderate plaques-free areas against coxsackie B1 virus, wild and attenuated strains of poliovirus types and different strains of echoviruses. Further quantitative studies were made with coxsackie B1 virus. In the PRT, the maximal tolerated dose gave 100 per cent plaque reduction for the com-

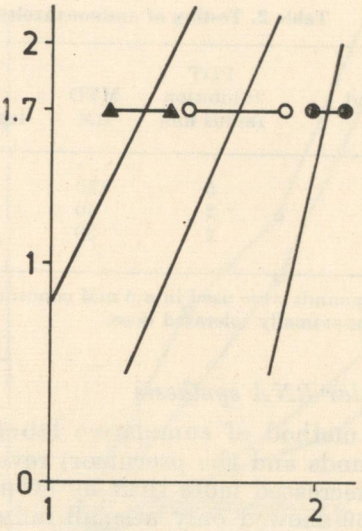

Fig. 2.
Regression straight lines of Z 190/69 (●), Z 40/70 (○) and Z 12/73 (▲) with estimated 95% confidence intervals for the concentrations giving an inhibition of 1.7 log unit (98%)
Abscissa: 10^{-6} molar substance concentration in log units; ordinate: inhibition in log units

pounds Z 190/69 and Z 12/73 and 95 per cent plaque reduction for Z 40/70 (Fig. 1). The virus-induced CPE was nearly completely suppressed by the compounds Z 190/69 and Z 12/73 (Table 1) during a time course of 10 replication cycles of coxsackie B1 virus. In one-step growth cycle experiments, the infectious virus yield was inhibited by the maximal tolerated dose by about 98.2—99.9 per cent (Table 2). Dose-action curves were estimated and the regression straight lines obtained are shown in Fig. 2.

Mode of action

One of the compounds tested (Z 190/69) exerted neither a virucidal effect on coxsackie B1 virions nor a significant influence on virus adsorption and penetration prosesses (Table 3).

Table 1. Turbidity measurements of coxsackie B1 virus-infected FL-cell suspension cultures (8×10^5 cells) untreated and treated with Z 190/69 and Z 12/13

Hours after infection	Extinction at 650 nm			
	100 μM Z 190/69	25 μM Z 12/73	Virus control	Cell control
0	0.345	0.349	0.350	0.354
20	0.349	0.356	0.481	0.359
40	0.354	0.362	0.548	0.363
60	0.367	0.373	0.571	0.369
80	0.377	0.384	0.583	0.379

Mean values of 9 determinations

Table 2. Testing of aminooxazoles against coxsackie B1 virus in FL cells

Compound	PDT Inhibition radius mm	MTD μM	Inhibition of virus yield by MTD $\log_{10}$ units	%	Inhibition of 1.7 $\log_{10}$ units with confidence intervals μM in $\log_{10}$
Z 190/69	5	120	3.1	> 99.9	2.03 ± 0.04
Z 40/70	2	50	1.75	98.2	1.71 ± 0.17
Z 12/73	2	30	2.12	99.2	1.35 ± 0.12

The compounds were used in a 5 mM concentration, which showed no toxicity in PDT.
MTD = maximally tolerated dose.

Cellular RNA synthesis

The method of cumulative labelling (simultaneous addition of the test compounds and the precursor) revealed that ^{3}H-uridine incorporation into RNA decreased more than by 50 per cent with Z 12/73 and Z 40/70, while Z 190/69 showed only a small influence of the incorporation rate (Fig. 3). Uridine is not a direct precursor of RNA synthesis because it must be taken up into cells and also phosphorylated to UTP before incorporation. The question arose as to whether the compounds influenced these two processes, transport and phosphorylation.

To solve this problem, the method of prelabelling is useful if the actions of the substance were expressed after ^{3}H-uridine had been taken up into the cells at 16 °C for 1 hr and also phosphorylated to a high extent. So far, we

Table 3. Testing of compound Z 190/69 for its virucidal effect and effect on adsorption and penetration of coxsackie B1 virus

Test for	PFU/ml Test	Control
Virucidal effect 7 hr incubation of cell-free virud without and with substance (120 μM). After virus adsorption, 3 washings — plaque assay	68, 69, 73	62, 67, 72
Effect on adsorption 1 hr adsorption at 4 °C without and with substance (120 μM), 3 washings — plaque assay	58, 63, 54	52, 57, 62
Effect on penetration 1 hr adsorption at 4 °C, 3 washings, penetration at 24 °C without and with substance (120 μM), 3 washings, addition of immune serum (about 500 neutralizing units) for 45 min at 37 °C, 3 washings — plaque assay	65, 62, 71	61, 64, 69

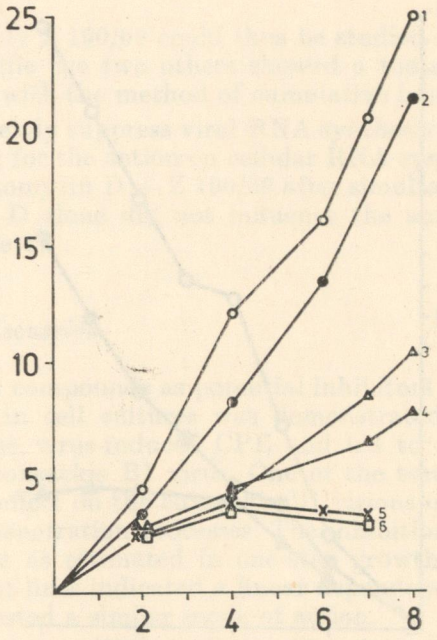

Fig. 3.

Incorporation of ^{3}H-uridine (0.5 μCi/ml per tube) into acid insoluble material from FL cells. Test compounds and uridine were added simultaneously at hr 0 (cumulative labelling)

Curves:
1 — Untreated cell control
2 — Z 190/69, 100 μM
3 — Z 12/73, 50 μM
4 — Z 40/70, 25 μM
5 — Actinomycin D, 0.2 μg/ml
6 — Z 190/69 (100 μM) and actinomycin D (0.2 μg/ml)

Abscissa: hr; ordinate: ^{3}H-uridine incorporation count/min × 10^{-2}

observed that Z 190/69 showed no influence on RNA synthesis after shift up to 37 °C using the prelabelled nucleotide pool and that the action of the other two compounds was much smaller than revealed by cumulative labelling (Fig. 4).

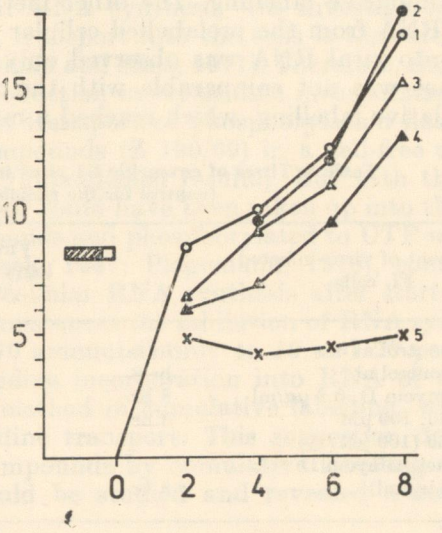

Fig. 4.

Incorporation of ^{3}H-uridine into cellular RNA of FL cells after prelabellin with ^{3}H-uridine (1 μCi/0.5 ml per tube) for 1 hr at 16 °C (shaded column). Empty column: washing period. Substances added at time 0.

Abscissa: hr of incubation at 37 °C; ordinate: ^{3}H-uridine incorporation count/min × 10^{-2}

Designation of curves as in Fig. 3.

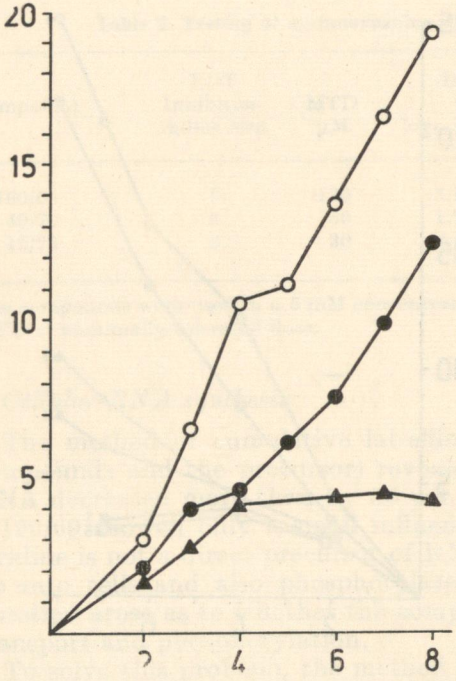

Fig. 5.
Incorporation of ^{3}H-uridine (0.5 μCi/ml per tube) into acid-insoluble material from coxsackie B1-infected FL cells without (○) and with (●) 0.2 μg/ml actinomycin D, after infection under conditions of cumulative labeling. Z 190/69 (100 μM) and actinomycin D (▲)
Abscissa: hr post infection; ordinate: ^{3}H-uridine incorporation count/min × × 10^{-2}

Viral RNA synthesis

Both the cumulative and the prelabelling method were used. But viral RNA synthesis under the action of actinomycin D could only be demonstrated by cumulative labelling. The other method revealed no incorporation into viral RNA from the prelabelled cellular nucleotide pool. A small incorporation into viral RNA was observed only in one of 5 experiments. But this increase was not comparable with the viral RNA prepared in parallel by cumulative labelling, which reached a much higher value.

Table 4. Titres of coxsackie B1 virus in untreated and treated FL cells (control for the radiobiological assay)

Treatment of virus-infected FL cells		Virus titre $\log_{10}$ $TCID_{50}$/ /0.2 ml	Inhibition $\log_{10}$ units	Inhibition %
Virus control at	hr 0	4.67	—	—
Virus control at	hr 8	8.5	—	—
Actinomycin D, 0.2 μg/ml	8 hr	8.5	—	—
Z 190/69, 100 μM	8 hr	5.5	3	99.9
Z 190/69 (100 μM) + + actinomycin D (0.2 μg/ml)	8 hr	5.0	3.5	> 99.9

Of the compounds investigated only Z 190/69 could thus be studied for its action on viral RNA synthesis, while the two others showed a too strong depression of uridine incorporation with the method of cumulative labelling.

But Z 190/69 was found to completely suppress viral RNA synthesis under the action of actinomycin D (Fig. 5; for the action on cellular RNA synthesis of actinomycin D alone and of actinomycin D + Z 190/69 after simultaneous addition see Fig. 3). Actinomycin D alone did not influence the antiviral action of compound Z 190/69 (Table 4).

Discussion

A new class of synthetic antiviral compounds as potential inhibitors of the replication of coxsackie B1 virus in cell cultures was demonstrated. The compounds examined depressed the virus-induced CPE and led to nearly 100 per cent plaque reduction of coxsackie B1 virus. One of the test compounds neither showed a virucidal effect on the coxsackie B1 virions nor did it affect the virus adsorption and penetration processes. The inhibition took place in the virus replication cycle as estimated in one-step growth cycle experiments. The regression straight lines indicated a linear dependence and ran almost parallel. This also suggested a similar mode of action.

The test compounds were also active in PDT against wild and attenuated strains of poliovirus, coxsackie B3—B5 viruses and echoviruses 11, 30 and 33 (unpublished results). The antiviral spectrum of these compounds was also tested and a 100 per cent plaque reduction found with poliovirus types 1, 2 and 3, coxsackie A9 virus and echovirus 12 in another laboratory (Koroleva *et al.*, 1975).

The effect on cellular and viral RNA synthesis was investigated with ^{3}H-uridine as precursor. However, transport into the cells is the rate-limiting step in the incorporation (Plageman and Shea, 1971). Therefore, since two aminooxazoles showed a marked reduction in ^{3}H-uridine incorporation into RNA, the question of inhibition of transport or phosphorylation has to be discussed. Testing of one of the compounds (Z 190/69) in a cell-free uridine kinase system revealed no action (unpublished results). But with the prelavelling method, when the precursor could have been taken up into the cells without influence of the test compounds and phosphorylated to UTP without incorporation into RNA (Scholtissek, 1967; Plagemann, 1970), compound Z 190/69 showed no influence on cellular RNA synthesis after starting by shift up to 37 °C. Under these circumstances the inhibition of RNA synthesis by compounds Z 12/73 and Z 40/70 amounted only to 10 and 20 per cent, respectively. The reduction of uridine incorporation into RNA of 60 and 70%, respectively, shown by the method of cumulative labelling, was possibly caused by inhibition of uridine transport. This suggestion does not allow an investigation of these compounds by cumulative labelling. Therefore, only substance Z 190/69 could be studied and revealed a complete inhibition of viral RNA synthesis.

The available data make it impossible to decide whether the lack of ^{3}H-uridine incorporation from the prelabelled nucleotide pool into coxsackie B1 virus RNA was caused by the presence of two different nucleotide pools as postulated by Plagemann (1971).

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