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MECHANISM OF ACTION OF ANTIVIRAL HETARYLHYDRAZONES IN VITRO

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Summary. — Three antiviral hetarylhydrazones showed a depression of ^{3}H-uridine incorporation into RNA of FL cells as a consequence of inhibition of uridine transport into the cells. By eliminating the action of the compounds on transport processes with the prelabelling method, only one compound showed a nearly 50% reduction of cellular RNA synthesis during the whole incubation period, whereas the other two compounds had no influence during the first 6 hr. Under prelabelling conditions all three compounds completely inhibited the synthesis of Mengo virus RNA. One compound did not affect cellular DNA synthesis, but presumably depressed thymidine transport into the cells.

Key words: Mengo virus; hetarylhydrazones; inhibition of uridine transport; inhibition of viral RNA synthesis

Introduction

The antiviral activity of hetarylhydrazones against Mengovirus (genus *Cardiovirus*) in vivo and in vitro was described (Veckenstedt and Ulbricht, 1977; Veckenstedt, 1978; Veckenstedt *et al.*, 1979; Tonew *et al.*, to be published). The present paper deals with the mode of action in vitro as monitored by radiobiological experiments.

Material and Methods

Cell cultures. Cultivation of FL cells and media were described by Tonew and Tonew (1971). L cells were grown and maintained in Eagle's minimal essential medium (MEM) with 10 and 2% inactivated calf serum, respectively.

Virus. Mengo virus and its cultivation and passages were described by Tonew and Tonew (1971) and Tonew and Bleecken (1971).

Chemicals. Z 98/69: 3-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenyloxazolyl-(2)-hydrazone], Z 124/73: 3-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenylthiazolyl-(2)-hydrazone] and Z 38/74; 4-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenylthiazolyl-(2)-hydrazone] were synthesized and kindly provided by Dr. H. Ulbricht from our Institute (Ulbricht *et al.* 1971*a*, *b*). Stock solutions were prepared in methylglycol and further dilutied in maintenance medium. Actinomycin D from our Institute, sodium deoxycholate (DOC) from VEB Chemisches Werk, Berlin-Grünau, GDR; filter disks of chromatography paper FN 7, 90-100/30 min, from VEB Spezialpapierfabrik Niederschlag/Erzgebirge, GDR; ^{3}H-5-uridine from the

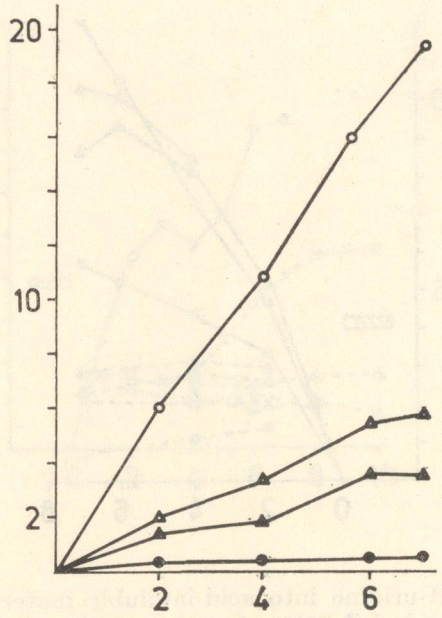

Fig. 1.

Influence of hetarylhydrazones on ^{3}H-uridine incorporation into acid-insoluble material of FL cells Test compounds and ^{3}H-uridine were added simultaneously (cumulative labelling).
Control (○); Z 98/69 50 μM (●) Z 124/73 30 μM (△) Z 38/74 50 μM (▲).
Abscissa: time in hr;
ordinate: ^{3}H-uridine count/min × 10^{-2}

Radiochemical Centre, Amersham, England (specific activity 903 GBq/mmol); ^{3}H-thymidine (spec. activity 814 MBq/mmol and ^{14}C-valine (spec. activity 4.6 GBq/mmol) from the Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia; and PPO and dimethyl-POPOP from Packard Instrument International SA, Zürich, Switzerland for the toluene scintillator fluid were used.

Incorporation of ^{3}H-5-uridine. Cumulative labelling. Cells were labelled with 18.5 kBq uridine per tube in the presence of test compounds. The radioactivity in the acid-soluble material was determined at the indicated times after incubation at 37 °C. Three tubes of each group were collected, the medium replaced by 0.15 % DOC. The cells were shaken off from the glass wall for nearly 5 min, precipitated with 6 % trichloroacetic acid, collected on filter disks and washed in the usual manner (trichloroacetic acid, ethanol, ether). Radioactivity was measured in toluene scintillator fluid with a Packard liquid scintillation spectrometer. *Prelabelling method.* Cells were labelled with uridine (37 kBq per tube) and, if necessary, simultaneously infected with virus for 1 hr at 16 °C (Tonew and Tonew, 1974). Surplus of ^{3}H-uridine and unadsorbed virus were washed out. Thereafter test compounds were added with medium and the cell cultures were shifted up to 37 °C for further incubation. Tubes were collected and radioactivity was estimated as described above.

Incorporation of ^{3}H-thymidine and ^{14}C-valine. Cumulative labelling and prelabelling experiments were carried out as described above The preparation of test tubes was also the same. For experiments with labelled valine, serum-free Eagle's MEM without valine was used, for the other nucleosides Eagle's MEM without serum.

Results

Effect of test compounds on ^{3}H-uridine incorporation into cellular RNA of FL cells

Cumulative labelling. Simultaneous addition of the three hetarylhydrazones tested with the labelled nucleoside revealed a strongly depressed incorporation

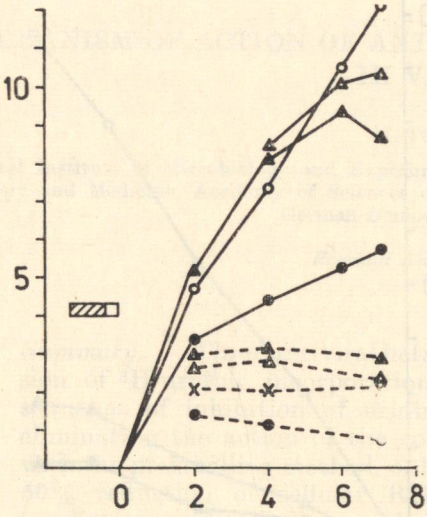

Fig. 2.

^{3}H-uridine incorporation into cellular RNA after prelabelling of FL cells for 1 hr at 16 °C
Test substances were applied at time 0 after washing out the excess of ^{3}H-uridine. Symbols and substance concentrations the same as in Fig. 1. Dotted lines designate actinomycin D alone (×) or in combination with test compounds.
Shaded bar: prelabelling for 1 hr at 16 °C; empty bar: washing period at 4 °C.
Abscissa: time in hr; ordinate: ^{3}H-uridine count/min × 10^{-2}

of ^{3}H-uridine into acid-insoluble material of FL cells. In the presence of compound Z 98/69, nearly no ^{3}-H uridine was incorporated for 7 hr (Fig. 1).

Prelabelling method. Another result was obtained when the cells were pretreated with ^{3}H-uridine to fill up the soluble nucleotide pool at low temperature, incorporation into RNA under such conditions being negligible, and the compounds were applied afterwards. After prelabelling, excess uridine was washed out, and after addition of test compounds the shift up to 37 °C allowed incorporation from the prelabelled nucleotides into RNA. Compounds Z 124/73 and Z 38/74 did not affect cellular RNA synthesis for nearly 6 hr, while from 6 to 8 hr a decreased synthesis rate was observed. In the presence of Z 98/69, cellular RNA synthesis was depressed nearly to 50 % for the whole incubation period (Fig. 2).

Effect of test compounds on ^{3}H-uridine incorporation into viral RNA

From the results presented above it was obvious that only the method of prelabelling is useful to investigate the influence of the test compounds on viral RNA synthesis. Actinomycin D was used to decrease cellular RNA synthesis. The combined action of actinomycin D and the compounds is illustrated in Fig. 2.

^{3}H-Uridine incorporation into untreated and actinomycin D-treated virus-infected cultures is shown in Fig. 3. The increased incorporation of ^{3}H-uridine after 4 hr of incubation in the presence of actinomycin D revealed the actual virus-induced RNA synthesis. This RNA-dependent RNA synthesis was inhibited by all three compounds tested. With two of them the observation period lasted up to ten hours, but no viral RNA synthesis had started.

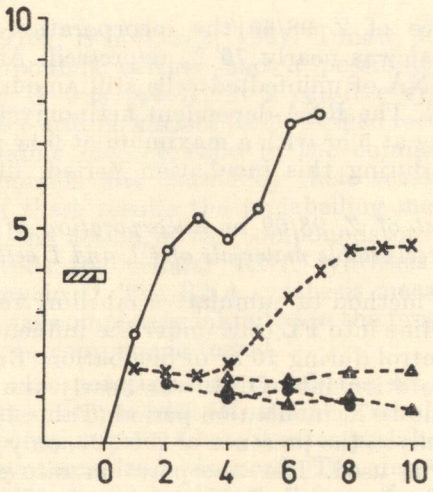

Fig. 3.

^{3}H-uridine incorporation into RNA of virus-infected cells after prelabelling without (virus control, ○) and with (×) actinomycin D

Test compounds were added simultaneously with actinomycin D as in Fig. 2 (symbols the same as in Fig. 2). Abscissa: time in hr; ordinate: ^{3}H-uridine count/min × 10^{-2}

Experiments on L cells

The mode of action of Z 98/69 on L cells, uninfected and infected with Mengo virus, was nearly the same as in FL cells. The inhibition of the infectious virus yield during one replication cycle was somewhat smaller in comparison with FL cells so that for L cells a higher concentration (75 μM was used (200 and 75 μM: 2.55 and 1.58 log inhibition, respectively). In the

Table 1. Action of Z 98/69 on ^{3}H-thymidine and ^{14}C-valine incorporation into acid-precipitable material of FL and L cells

Time of incubation hr	Count/min in % of control		
	^{3}H-Thymidine, FL cells		^{14}C-valine, L cells
	Cumulative labelling 7.4 KBq/ml	Prelabelling 37 KBq/0.5 ml	Cumulative labelling 3.7 KBq/ml
2	2.7	113	8.3
3	4.2	80	
4	2.2	104	5.3
5	2.0	81	
6	2.8	116	6.1
7	2.2	94	
8	2.3	86	4.3
9	1.6	96	5.1
10	2.1	125	

Concentration of Z 98/69 was 50 μM for FL and 75 μM for L cells.

presence of Z 98/69 the incorporation of ^{3}H-uridine into acid-insoluble material was nearly 75 % depressed. After prelabelling, the incorporation into RNA of uninfedted cells still amounted to 50 % in comparison to the control. The RNA-dependent actinomycin D-resistant viral RNA synthesis starting at 5 hr with a maximum at 9 hr p. i. was completely depressed by Z 98/69 during this incubation period, like in FL cells (data not shown).

Effect of Z 98/69 on incorporation of ^{3}H-thymidine and ^{14}C-valine into acid-precipitable materials of FL and L cells, respectively

The method of cumulative labelling revealed only small incorporation of thymidine into FL cells under the influence of Z 98/69, namely about 3 % of the control during 10 hr of incubation. But with the prelabelling method the incorporation into DNA was nearly the same as in the untreated control over the 10 hr incubation period (Table 1). To estimate the protein synthesis in L cells in the presence of Z 98/69, only the method of cumulative labelling could be used. The incorporation rate of labelled valine into L cells was strongly depressed (Table 1).

Discussion

Results concerning antiviral activity of hetarylhydrazones on Mengo virus will be described (Tonew *et al.*, in preparation). The present investigations were aimed at elucidating their action on cellular and viral RNA synthesis as well as on cellular DNA synthesis.

The method of cumulative labelling revealed with all three compounds tested a strongly diminished incorporation of ^{3}H-uridine into acid-insoluble material. However, uridine is not a direct precursor of RNA. It must be taken up into cells and phosporylated in UTP before incorporation into RNA can take place. Therefore the rate-limiting step for incorporation of uridine into RNA is the transmembraneous transport and also phosporlyation to nucleotides (Scholtissek, 1968; Steck *et al.*, 1969; Plagemann and Roth, 1969; Plagemann, 1970*b*; Plagemann and Shea, 1971).

To overcome the problem of influencing uridine transport and phosphorylation by test substances, the method of prelabelling was introduced (Tonew and Tonew, 1974). Test compounds were added after enrichment of the soluble nucleotide pool of cells by incubation with ^{3}H-uridine for only 1 hr at 16 °C, when no RNA synthesis takes place. Just before starting RNA synthesis by shifting up to 37 °C, test compounds were added and their direct action on RNA synthesis could be observed. With Z 124/73 and Z 38/64 incorporation of ^{3}H-uridine was not inhibited for up to 6 hr; afterwards the incorporation rate was lowered. With compound Z 98/69, a 50 % decrease in incorporation was observed during the whole incubation period. These results have to be interpreted as an actual inhibition of cellular RNA synthesis, because with the method of prelabelling the compounds cannot act on uridine transport into cells. After prelabelling, most of uridine is present

in the form of UTP (Plagemann, 1970*a*; Scholtissek, 1967; Tonew, unpublished results). Therefore the compounds neither had a possibility to influence the phosphorylation steps. It was proved in a cell-free system that Z 124/73 and Z 38/74 do not impair uridine kinases (unpublished results). Probably the strong decrease of uridine incorporation in the cumulative experiments by the three test compounds was caused by their action on uridine transport. In consequcnce of these results the prelabelling method was the only choice in investigating the action of the compounds on viral RNA synthesis. In virus-infected cells the cellular RNA synthesis was depressed by the addition of actinomycin D. The RNA synthesis measured after 4 hr p. i. was virus-induced and was completely inhibited in the presence of actinomycin D and any of the three compounds tested.

The results concerning thymidine incorporation into uninfected FL cells were comparable to and nearly the same as those with ^{3}H-uridine. Cumulative labelling resulted in a lowered incorporation rate, whereas after prelabelling no influence of Z 98/69 on DNA synthesis was observed. The depressed incorporation in the presence of Z 98/69 should be discussed from the point of view of inhibition of thymidine transport into the cells. But further experiments from this aspect were not carried out.

In the presence of Z 98/69, the incorporation of valine into L cells was also inhibited. The method of prelabelling did not work with labelled amino acids (unpublished results). Therefore no conclusion about inhibition of protein synthesis was possible from this experiment.

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