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ANTIVIRAL ACTIVITY OF HETARYLHYDRAZONES

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Summary. — Some hetarylhydrazones showed antiviral activity against Mengo virus in vitro. The replication of influenza A and B viruses was affected neither in vitro (chick chorioallantoic membranes) nor in vivo (mice). Based on dose response curves of the active hetarylhydrazones the compound Z 98/69 appeared most effective in vitro. This compound neither inactivated the extracellular virus nor inhibited its adsorption and penetration; it reduced virus replication when added 1-3 hr after Mengo virus inoculation. As shown by plaque reduction test vaccinia virus was also inhibited. A partial inhibition of rhinovirus 1B multiplication was observed whereas other picornaviruses were not affected.

Key words: *antiviral action in vitro*; *Mengo virus*; *picornaviruses*; *influenza viruses*; *hetarylhydrazones*

Introduction

The antiviral activity of 3-[bis-(2-hydroxyethyl-amino]-acetophenone--[4,5-diphenyl-oxazolyl-(2)]-hydrazone (ZIMET 98/69) and related compounds against lethal Mengo virus encephalitis in mice was described in detail by Veckenstedt and Ulbricht (1977) and Veckenstedt (1978). A further report showed that the substance ZIMET 98/69 inhibited certain RNA virus infections in mice such as encephalomyocarditis (EMC), Columbia SK, Martin Mahoney (MM), Semliki forest and vaccinia viruses (Veckenstedt *et al.*, 1979). The hetarylhydrazones had been discovered in the course of an antiviral screening programme using Mengo virus. Their activity in vitro was analysed for several years; the results are presented in this paper.

Materials and Methods

Cell cultures. FL and chick embryo cells (CEC) were prepared and cultured as described (Tonew and Tonew, 1969, 1971). Chorioallantoic membrane (CAM) culture was prepared according to

Table 1. Chemical composition of the ZIMET compounds

Designation	Composition
Z 68/72	3-aminoacetophenone-[4,5-diphenyl-oxazolyl-(2)-hydrazone]
Z 152/73	3-(carboxymethyleneamino)-acetophenone-[4,5-diphenyl-oxazolyl--(2)-hydrazone]
Z 98/69	3-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenyl--oxazolyl-(2)-hydrazone]
Z 124/73	3-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenyl--thiazolyl-(2)-hydrazone]
Z 46/69	3-[bis-(2-hydroxyethyl)-amino]-acetophenone-[benzimidazolyl-(2)--hydrazone]
Z 67/72	4-aminoacetophenone-[4,5-diphenyl-oxazolyl-(2)-hydrazone]
Z 418/68	4-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenyl--oxazolyl-(2)-hydrazone]
Z 38/74	4-[bis-(2-hydroxyethyl)-amino]-acetophenone-[4,5-diphenyl--thiazolyl-(2)-hydrazone]
Z 417/68	4-methylnitrosamino-benzaldehyde-[4,5-diphenyl-oxazolyl-(2)--hydrazone]
Z 45/73	4-methylamino-benzaldehyde-[4,5-diphenyl-oxazolyl-(2)-hydrazone]
Z 44/69	4-methylnitrosamino-benzaldehyde-[benzimidazolyl-(2)-hydrazone]

Horwath (1954). HeLa cells* (Bristol) were grown in Eagles minimal medium (MEM) supplemented with 10% calf serum. Cultivation of human embryonal fibroblasts was carried out as described by Schmidt and Mentel (1975).

Viruses. Mengo virus was passaged as previously reported (Tonew and Bleecken, 1971; Tonew and Tonew, 1971). In addition, following viruses were tested: Newcastle disease virus (NDV), the sheep abortion agent (Tonew and Tonew, 1969), polio, Coxsackie and ECHO viruses (Tonew *et al.*, 1971), EMC virus (Tonew *et al.*, 1977), vaccinia virus (strain Berlin), pseudorabies virus (strain BUK), fowl plague virus (FPV, strain Dobson), Sindbis virus (Tonew *et al.*, 1974), rhinovirus type 1 B, influenzavirus A strain England 42/72 (H3N2) and B (Tokyo 7/66) (Kanel and Indulen, 1974). The latter two strains were adapted to mice by lung passages.

Virus assays. Agar difusion plaque inhibition test (ADPI) and plaque reduction test were used as described (Rada and Závada, 1962; Tonew and Tonew, 1969). The plaque reduction test with the rhinovirus was carried out by adding the compound into the overlay in compatible concentrations of 50 µmol/l or lower; HeLa cells were incubated for 60 to 72 hr.

The overlay for testing of rhinovirus consisted of MEM (5 times concentrated) 20 ml, calf serum (inactivated 3 × 30 min 56° C) 10 ml, $NaHCO_3$ (7.5% solution) 2.2 ml, antibiotics (streptomycin 100 µg/ml and penicillin 100 IU/ml) 1 ml, glutamine solution (3%) 1 ml, DEAE dextran (0.2%) 3 ml, $MgCl_2 \times 12\ H_2O$ (24.4 g in 100 ml H_2O) 5 ml, bidest. water 50 ml, agarose (1.2% solution) 50 ml, bidest. water up to 150 ml.

The influence of compounds on extracellular virus, virus adsorption, penetration and replication of Mengo virus was tested as reported (Tonew and Tonew, 1971; Tonew and Klimke, 1974). Titre determinations were carried out according to Reed and Muench (1938).

The inhibition of cytopathic effect (CPE) was examined under conditions of one step growth experiment: after inoculation of virus at about 10 $TCID_{50}$/cell, unadsorbed virus was washed out after 40 min at 37° C; each substance was added in the maintenance medium.

One step growth experiments were used for the timing of compound action as well as for the comparison of activity of the compounds on the base of an arbitrary limit. Briefly, compounds were added immediately after infection in different concentrations. From the inhibition of infectious virus yield at the end of replication cycle, the dose response curves were obtained. Then the

*The cell line was kindly supplied by Dr. A. Galabov, Department of Virology, Medical Academy, Sofia (Bulgaria)

Table 2. Antiviral activity of hetarylhydrazones on Mengo virus

Compound	Screening test* ADPI	One step replication MTD** mmol/l	Inhibition of virus yield by MTD $\log_{10}$ $TCID_{50}$	Concentrations causing 1.7 log inhibition log μmol/l and confidence interval
Z 68/72	∅			
Z 152/73	+	200	3.0	0.81 ± 0.13
Z 98/69	+	250	4.0	0.53 ± 0.08
Z 124/73	+	50	2.0	1.7
Z 46/69	∅			
Z 67/72	∅			
Z 418/68	+	50	1.25	> 1.7
Z 38/74	+	100	3.0	1.49 ± 0.05
Z 417/68	+	200	1.05	> 2.3
Z 45/73	∅			
Z 44/69	+	200	0.42	

* Drug concentration 5 mmol/l.
** Maximally tolerated dose.

regression straight lines were calculated and their linearity was examined. Doses causing virus yield inhibition of 1.7 log (98%) were calculated by computer (Tonew *et al.*, 1974).

Testing the antiviral action of the substance Z 98/69 to rhinovirus, the titres in treated and untreated cultures were compared at indicated times. The virus content was estimated according to Reed and Muench (1938) after 7 days incubation in human embryonal fibroblasts (Oehring and Schmidt, 1978).

Influenza viruses were cultivated at defined multiplicity in CAM in the presence or absence of the compounds. The titre difference determined the action of the compound (Indulen *et al.*, 1972).

Mice. In vivo experiments were performed in outbred mice weighing 18—20g. From 5 to $10LD_{50}$ were administered intranasally under ether narcosis in 0.05 ml inoculum. The compounds were diluted by means of 0.05% tween 80 and applied subcutaneously in a single dose (1 mmol/kg) 2 hr before and after infection and later on twice per day for 4 consecutive days. Groups of control and treated animals consisted of 10 animals for influenza A and 24 for influenza B, respectively (Veckenstedt and Horn, 1976). Statistical analysis was carried out as described (Veckenstedt *et al.*, 1979).

Compounds. The drugs (listed in Table 1) were synthesized and kindly provided by Dr. Ulbricht from Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena (Ulbricht *et al.*, 1971*a*, *b*).

Results

Antiviral action of the compounds

Using the ADPI test for screening of the hetarylhydrazones (concentration 5 mmol/l) certain compounds revealed antiviral action against Mengo virus and one compound against vaccinia virus (Tables 2 and 3). Other viruses such as Coxsackie virus B1, NDV, FPV, Sindbis, pseudorabies virus and the sheep abortion agent were not affected. Compound Z 98/69 was tested against other picornaviruses. By means of ADPI test antiviral activity

Table 3. Action of compound Z 98/69 against different viruses

Virus family	Virus strain	ADPI test*	Inhibition of CPE**	Inhibition of virus yield μmol/l	$\log_{10}$ PFU
Picornaviridae	Mengo	+	+	50	3.67
	EMC	0	0		
	Rhino 1B			100	1.13
	Polio 1—3	0			
	Polio attenuated 1—3	0	0		
	Coxsackie B1, 2, 3, 4, 5	0	0		
	ECHO 6		0		
	11, 30	0	0		
	33	0	0		
Togaviridae	Sindbis	0			
Orthomyxoviridae	FPV	0			
	Influenza A/England 42/72			55	0***
	B/Tokyo 7/66			55	0***
Paramyxoviridae	NDV	0			
Poxviridae	Vaccinia	+			
Herpesviridae	Pseudorabies	0			

* Drug concentration 5 mmol/l
** Drug concentration 250 μmol/l
*** No inhibition in CAM test

against rhinovirus type 1B was observed; the plaque-free zone was 12—14 mm in diameter. No inhibition of plaque formation or that of the CPE were seen with EMC, poliovirus 1—3 wild and attenuated strains, Coxsackie viruses B2, 3, 4, 5 and ECHO viruses types 6, 11, 30 and 33 (Table 3). None of the hetarylhydrazones exhibited any inhibitory effect on influenza A (England 42/72) and B (Tokyo 7/66) viruses neither in CAM cultures nor in mice (Table 4).

Table 4. Action of hetarylhydrazones on influenza viruses A/England 42/72 and B/Tokyo 7/66 in mice

Compound	Influenza virus			
	A/England 42/72		B/Tokyo 7/66	
	Rate of protection %	Significance	Rate of protection %	Significance
Z 68/72	22.2	0	n.t.*	
Z 152/73	0		n.t.	
Z 124/73	25	0	57.1	0
Z 67/72	0		42.8	0
Z 418/68	11.1	0	n.t.	
Z 38/74	0		28.6	0
Z 417/68	22.2	0	n.t.	
Z 44/69	0		42.8	0

* n. t. = not tested.
Drug dose: 1 mmol/kg twice daily for 5 days.

Table 5. Action of Z 98/69 (100 μmol/l) on extracellular Mengo virus, its adsorption and penetration

Treatment	Extracellular virus 3 hr/37° C	Extracellular virus 24 hr/37° C	Adsorbed virus 1 hr/4° C	Penetrated virus 1 hr/24° C
Yes	45*	25	106	67
No	44	24	100	83

* Titre in PFU/ml

Quantitative investigations

Inhibition of plaque formation was followed quantitatively with two compounds. A 50% plaque reduction of Mengo virus was brought about by 30 μmol/l of Z 417/69 and 20 μmol/l of Z 98/69; inhibition of vaccinia virus occurred by 100 μmol/l of Z 98/69. At concentration of 50 μmol/l this compound reduced the plaque number of rhinovirus 1B by 95% and at concentration of 5 μmol/l by 38%, respectively.

With Mengo virus 6 out of 7 compounds showed a significant decrease of the infectious virus yield by 1.0 log or more ($\geq$ 90 %) when tested under growth conditions using maximally tolerated doses (Table 2). Dose response curves, comparing the activity of the compounds on the base of concentrations causing a defined inhibition of 1.7 log units (98%) of virus yield, showed that Z 98/69 was the most active compound. To achieve 98% reduction of virus multiplication, the compound Z 98/69 was active in the smallest concentration among all compounds tested (Table 2).

Timig of antiviral action

Investigations on the mode of action were carried out with the most active compound Z 98/69 in Mengo virus infected FL cells. Concentration of 100 μmol/l neither inactivated the extracellular virus nor influenced virus

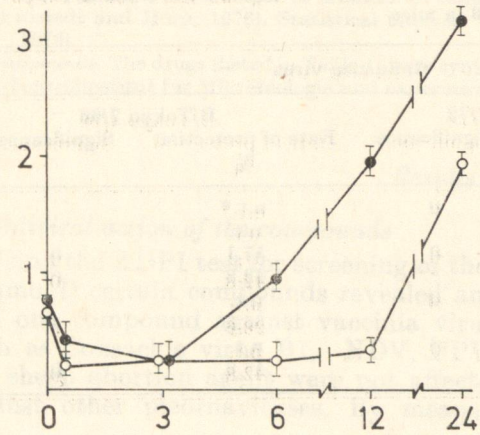

Fig. 1.
Antiviral effect of the compound Z 98/69 on replication of rhinovirus 1 B in human embryonal fibroblasts
Cells infected in the presence (○) and absence (●) of the drug
The drug (100 μmol/l) was added immediately after infection
Abscissa: hr p. i.
Ordinate: virus titre in $\log_{10}$ $TCID_{50}$/0.1 ml.

adsorption or penetration (Table 5). When added immediately after infection in concentration of 50 μmol/l, it reduced the virus yield by 3.54 log units (> 99.9 % reduction) under one step growth conditions within 8 hr. The inhibition was lower (2.09 log units = 99% reduction) when the compound was added 3 hr p. i. Administration of Z 98/69 at 5 hr. p. i. was without any effect on virus multiplication.

Replication of rhinovirus at 100 μmol/l concentration of Z 98/69 was reduced of 1.16 log units (statistical significance p = 99%). Administration of 50 μmol/l also resulted in a significant inhibition, whereas 25 μmol/l caused only slight reduction in one out of two experiments.

The time dependence curves showed a different course in treated cultures versus controls (Fig. 1). It is to be seen that the replication of rhinovirus type 1B was significantly inhibited from 6 till 24 hr p. i.

Discussion

Some hetarylhydrazones showed antiviral activity against Mengo virus as detected by plaque reduction test in vitro. The mode of action was studied with compound Z 98/69: no inactivation of the extracellular virus, no altered adsorption and penetration could be demostrated. One step growth curve showed reduced virus yield after addition of the compound from 0—3 hr p. i. It was shown recently that the viral RNA synthesis was inhibited by these compounds (Tonew, 1981).

Besides Mengo virus, only rhinovirus 1B and vaccinia virus exhibited sensitivity to Z 98/69. However, the inhibitory action of Z 98/69 on the replication of rhinovirus was not complete. When the compound was added immediately after infection, the depression of virus multuplication lasted only for 12 hr. Then the infectious virus yield increased in parallel with the untreated controls. The infectious titre of the treated probes was significantly lower than the titre of the untreated control at the cessation of replication by 24 hr.

The influence of hetarylhydrazones on influenza viruses A and B was investigated in CAM cultures in vitro as well as in vivo in mice. No antiviral effect could be found. The failure of Z 98/69 to act on influenza virus infection in mice was already reported (Veckenstedt *et al.*, 1979).

The antiviral activity of hetarylhydrazones on lethal Mengo virus encephalitis in mice was described previously (Veckenstedt and Ulbricht, 1977). Comparison of the rate of protection showed that Z 98/69 was the most effective compound (Veckenstedt, 1978). The same conclusion could be stated from in vitro data. Z 98/69 acts very early in virus replication as shown by further comparison of in vivo and in vitro experiments. However it is impossible to give any explanation of the mechanism of action in vivo on the base of the in vitro results with UZ 98/69, because there are too many factors which may influence the action of the compound in vivo.

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