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ON THE ANTIVIRAL ACTIVITY OF DIFFUSOMYCIN (OXAZOLOMYCIN)

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Summary. – The effect of the β-lactone antibiotic diffusomycin (oxazolomycin) was investigated against vaccinia (Lister), herpes simplex type 1 (Kupka), influenza A (WSN; H_1N_1), and Coxsackie A9 viruses. Diffusomycin reduced significantly the plaque formation of enveloped DNA and RNA viruses by more than 90 % in the range of the maximally tolerated dose. As could be shown with vaccinia virus, the antiviral action was not caused by virucidal effect on virions or by interaction with virus adsorption and penetration. In one-step growth cycle assays diffusomycin prevented the replication of herpes simplex type 1, vaccinia and influenza A viruses in a dose-dependent manner. The replication of influenza A viruses was blocked immediately after addition of the compound during zero to six hr p.i. Partial reversibility of the antiviral action was established by washing off the antibiotic from chicken embryo cells (CEC) infected with influenza A virus. Finally, replication of Coxsackie A9 virus was not inhibited by diffusomycin. Electron-optical studies revealed a reduced synthesis of HSV-1 nucleocapsids in dependence on the concentration of the compound.

Key words: diffusomycin; vaccinia virus; herpes simplex virus type 1; influenzavirus A (WSN); in vitro inhibition; virus replication

Introduction

Numerous antibiotics have been investigated on their antiviral activities *in vitro*. However, only few of them combine properties such as high antiviral activity and low toxicity *in vivo*. Diffusomycin was isolated from cultures of *Streptomyces albus* (JA 3453) and found identical with oxazolomycin (Mori *et al.*, 1985) when comparing the relevant high-field ^{1}H and ^{13}C NMR data (Gräfe *et al.*, 1988*a*, 1988*b*). The drug possesses promising antitumour and antibacterial properties (Mori *et al.*, 1985), but no information has been given with regard to any other biological activities so far, neither was its antiviral action investigated. In this paper we report on the antiviral activity of diffusomycin against influenza A, vaccinia and herpes simplex viruses *in vitro*.

Materials and Methods

Substance. Diffusomycin (oxazolomycin) was isolated and purified to homogenity as described (Gräfe *et al.*, 1988*a*); its chemical structure is shown in Fig. 1.

Cell cultures. Chicken embryo cells (CEC) were cultivated in Hanks solution with 10 % Parker 199 medium and 5 % calf serum (Tonew and Tonew, 1969). RH cells (continuous human kidney cell line) were grown in a medium consisting of one part Eagle's MEM and one part Hanks solution with 0.5 % lactalbuminhydrolysate with 5 % calf serum (Tonew and Glück, 1986). Human fibroblast cells were cultured with Eagle's MEM containing 5 % neonatal calf serum (SIFIN, Berlin).

Viruses. Vaccinia virus (strain Lister), herpes simplex virus type 1 (HSV-1) (strain Kupka), influenza virus A/WSN (H_1N_1) were propagated in CEC and RH cells, Coxsackievirus type A9 in human fibroblast cells.

Maximally tolerated dose (MTD). MTD was determined by microscopic observations as the compound concentration which caused no morphological alterations of cultured cells up to day 5 after treatment.

Antiviral activity. Assay for virucidity, influence on adsorption and penetration processes have been described earlier (Tonew and Tonew, 1971).

Plaque reduction test. The test was carried out without and with the test compound in different concentrations in an agar overlay as described by Tonew and Tonew (1969).

One step growth cycle experiments (OSGE). Using a m.o.i. of 20 PFU/cell and after washing off the nonadsorbed virus (three times), the infectious virus yield was determined according to Reed and Muench (1938) at different drug concentrations and in its absence by the end of the replication cycle (vaccinia virus and HSV-1 by 24 hrs, influenza virus WSN by 8 hrs p.i.).

Reversal of the antiviral activity. After infection with a m.o.i. of about 20 PFU/cell of influenza virus A/WSN in the presence of 15.75 μg/ml diffusomycin further procedures were carried out according to Tonew *et al.* (1975).

Microtitration assay. Coxsackie virus type A9 in different dilutions was checked against the inhibitor with MTD as well as with decreased concentrations in human fibroblast cells grown on titertek plates (Flow). The titre of untreated as well as antibiotic treated virus-infected cell cultures was determined according to Reed and Muench (1938).

Electron-optical examinations. Investigations were performed during OSGE in CEC infected with HSV type 1 in the presence of the antibiotic. Cell pellets were embedded and cut using the Reichert Wien ultramicrotome apparatus as reported (Zöpel *et al.*, 1973) and after contrasting viewed in Siemens Elmiscope 1 at 80 kV.

Results

The MTD of diffusomycin for CEC and RH cells was 125 μg/ml on day 5, which caused 88 to 100 % decrease of the plaque number of the three enve-

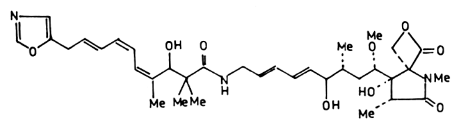

Fig. 1
Structure formula of diffusomycin (oxazolomycin)

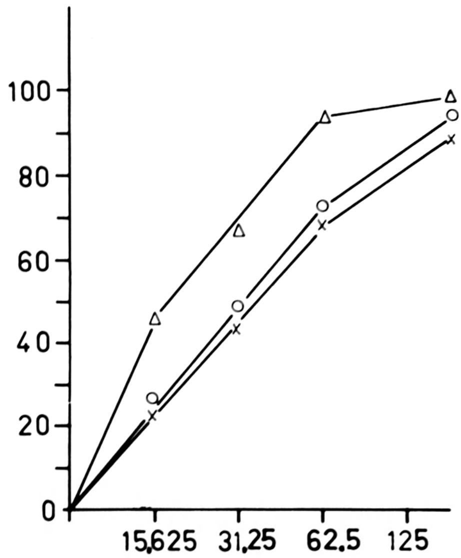

Fig. 2
Plaque reduction of HSV-1 (△), influenza (○) and vaccinia (x) viruses in CEC in dependence on the concentrations of diffusomycin (72 hr p.i.)
Ordinate: plaque reduction in per cent; abscissa: concentration in μg/ml

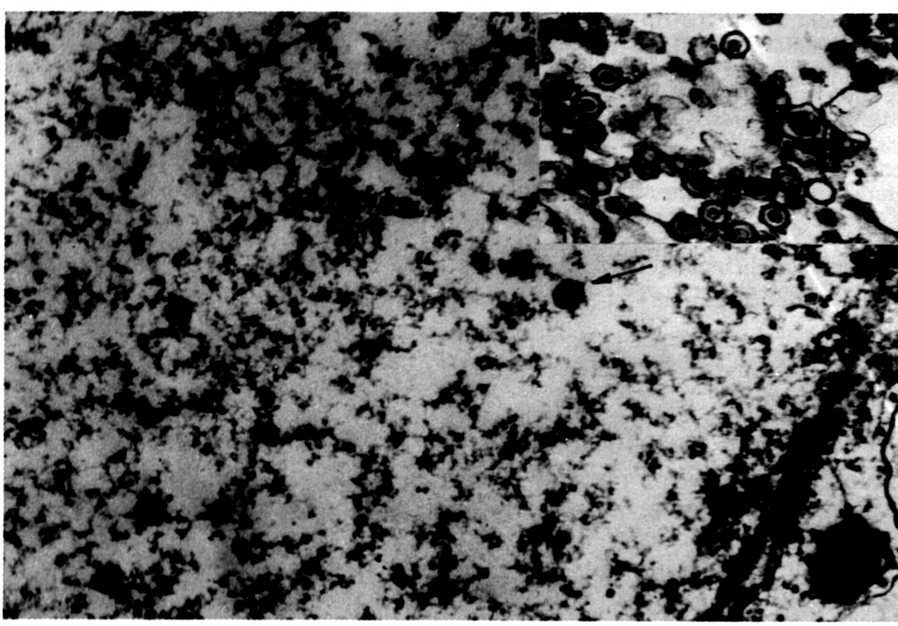

Fig. 3
HSV-1-infected CEC treated with 31.25 μg/ml diffusomycin. Occurence of a few nucleocapsids in the nucleus
Insert: nucleus of untreated virus-infected control cell.
Magnification 60,000 x

Table 1. Inhibition of virus replication by diffusomycin in one-step growth cycle experiments using RH cells and CEC (m.o.i. 20 $TCID_{50}$/cell)

Concentration of diffusomycin in $\mu g/ml$	Influenza A virus		Vaccinia virus Titre in $\log_{10} TCID_{50}/ml$		Herpes simplex virus type 1	
	RH cells	CEC	RH cells	CEC	RH cells	CEC
125	4.2 (>99.9)*)	3.7 (>99.9)	3.53 (>99.9)	2.7 (>99.9)	2.14 (>99.9)	2.37 (>99.9)
62.5	4.2 (>99.9)	4.2 (>99.9)	4.2 (>99.7)	2.92 (>99.9)	2.66 (>99.9)	2.7 (>99.9)
31.25	5.04 (>99.9)	5.63 (>99.9)	5.27 (96.3)	4.92 (99)	3.94 (>99.9)	3.93 (>99.9)
15.62	6.2 (99.7)	5.87 (99.9)	5.87 (85.2)	5.96 (89)	5.66 (99.7)	5.2 (>99.9)
0	8.7	8.87	6.7	6.92	8.14	8.37

*) In parenthesis: per cent inhibition

Table 2. Lack of activity of diffusomycin of cell-free virus particles and on the adsorption and penetration of vaccinia virus in CEC

Concentration in μg/ml	Cell-free virus Titre in $\log_{10}$ $TCID_{50}$/ml after incubation at 37 °C		PFU after treatment during		
	4 hrs	20 hrs	adsorption	penetration with immunoserum	penetration without immunoserum
125	6.7	5.92	121	114	129
0	6.87	6.18	137	126	141

loped viruses checked in CEC. A significant reduction was yet observed with 31.25 μg/ml diffusomycin (Fig. 2).

The drug in concentrations of 125 and 62.5 μg/ml reduced the infectious virus yield of influenza A, vaccinia and herpes simplex type 1 viruses during OSGE in both human and chicken cells by more than 99 % (Table 1).

The antibiotic showed neither virucidal effect nor an influence on virus adsorption and penetration processes as could be demonstrated with vaccinia viruses (Table 2). The mode of action was further studied only with influenza virus WSN. Infectious virus production was inhibited after application of the compound at zero as well as at 4 and 6 hr p.i. (Table 3). Removal of the antibiotic after 3 hr partially restored virus replication in a time depending manner. Haemagglutinin synthesis started with a few hours delay when the antibiotic treatment was shorter than 4 hr (Table 4). The antibiotic did not interfere with replication of Coxsackie virus type A9 in human fibroblast cells as tested by

Table 3. Antiviral activity of diffusomycin (125 μg/ml) against influenza virus A in CEC when added at 0.4 and 6 hr p.i.

Substance application at hrs	HAU/ml with diffusomycin	HAU/ml without diffusomycin	Virus yield in $\log_{10}$ $TCID_{50}$/ml with diffusomycin	Virus yield in $\log_{10}$ $TCID_{50}$/ml without diffusomycin
0	4	4	2.92	3.37
4	4	8	3.39	3.86
6	64	128	6.87	7.37*

* Titre of the untreated virus control at 8 hr p.i. was 8.87 $\log_{10}$ $TCID_{50}$/ml.
Titres were determined by 8 hr after addition of the compound.

Table 4. Reversion of the antiviral activity of diffusomycin (15.62 μg/ml) in dependence on the time of removal of the antibiotic

Duration of compound treatment in hrs	Hours after virus infection				
	0	4	8 HAU/ml	24	48
2	n.t.	2	16	128	256
3	n.t.	4	8	64	64
4	n.t.	4	8	16	32
O (Control)	<2	4	128	256	512

n.t. not tested

microtitration assays. The electron-optical investigations during replication of herpes simplex virus type 1 support the effect of the compound on virus replication. No virus particles could be observed in the presence of 62.5 μg/ml diffusomycin, whereas after application of 31.25 μg/ml only a few nucleocapsids appeared (Fig. 3).

Discussion

The β-lactone antibiotic diffusomycin (oxazolomycin) may represent a new structural type of antiviral agent. The antibiotic suppressed the replication of vaccinia, herpes simplex type 1, and influenza A viruses during OSGE both in CEC and RH cells. The mode of antiviral action of membranotropic antibiotics, in general, could involve inhibition of virus uncoating as an early stage of virus replication (Korant *et al.,* 1984). But diffusomycin apparently inhibited later stages of influenza A replication (about 4 to 6 hr p.i.) suggesting other yet unidentified ability of the drug acting during maturation and/or virus release. The absence of susceptibility of Coxsackie virus A9 appears to be an interesting fact which will promote further studies towards elucidation of the mode of antiviral action of diffusomycin.

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