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EFFECTS OF NORAKIN ON RESPIRATORY SYNCYTIAL VIRUS IN TISSUE CULTURE AND IN MICE

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Summary. — Norakin at 1 μg/ml inhibits the reproduction of respiratory syncytial virus (RSV) in Vero cells to 50 % and at 5 μg/ml to 90 %. The development of lung lesions in RSV-infected BALB/c mice was suppressed by 70 % when the animals were treated with two doses (25 mg/kg each) of norakin, 30 min before and 4 hr post infection (p.i.), respectively.

Key words: *respiratory syncytial virus*; *BALB/c mouse*, *norakin*

Introduction

Respiratory syncytial virus (RSV) causes severe bronchiolitis in infants and young children. Vaccine development has proved difficult (Kim *et al.*, 1969; Parrot *et al.*, 1975). Contrary to previous assumptions, RSV is not monotypic (Anderson *et al.*, 1985; Mufson *et al.*, 1985). The only antiviral agent shown to have efficacy in the therapy of human RSV infection is ribavirin (Hall *et al.*, 1983). Norakin is an anticholinergic anti-Parkinson drug which inhibits influenza and measles virus replication *in vitro* (Presber *et al.*, 1984) and is therapeutically effective in influenza virus-infected mice and chicken (Heider, 1986). We tested the effect of norakin against RSV replication *in vitro* as well as in a mouse model with promising results.

Materials and Methods

Virus. RS virus subtype A (strain Long) was grown in FL cells using Eagle's MEM supplemented with 2 % foetal calf serum (Staatliches Institut für Immunpräparate und Nährmedien, Berlin) and 0.3 % glutamin. Virus suspensions were stored at —196 °C.

Cell culture. FL and Vero cells were passaged with Eagle's MEM and 10 % neonatal calf serum. Toxicity tests on Vero cells were performed in 15 cm^2 flasks; virus was assayed on cells grown in 24-well Linbro plates (input 2×10^5 cells per well).

Compound. Norakin (1-tricyclo/2.2.1.$0^{2,6}$/ $hept^{-2-yl}$-1-phenyl-3-piperidino-propan-1-ol hydrochloride) was provided by VEB Fahlberg-List, Magdeburg.

Toxicity test. The appropriate amount of norakin stock solution (4 mg/ml in distilled water) was pipetted into culture flasks, immediately followed by $1-2 \times 10^5$ Vero cells in Eagle's MEM with 10 % neonatal calf serum. The cultures were incubated at 37 °C until the untreated controls became confluent. After trypsinization the cells were stained with trypan blue and counted. The number of cell divisions was calculated as

$$g = \frac{\log \frac{z}{z_o}}{\log 2}$$

where g denoted the number of generations, z the cell count and z_0 the input cell count. The antiviral therapeutic index was expressed as the quotient of the antiviral and the cytostatic ID_{50}s.

Antiviral activity in vitro. The compound was evaluated by a focus test. Virus and compound dilutions were mixed and added to Vero cell monolayers. Adsorption was allowed for 2 hr at 37 °C. The inoculum was then replaced by a 0.6 % methyl cellulose overlay containing Eagle's MEM, 5 % foetal calf serum and the test compound. The plates were incubated for 7 days at 37 °C in a CO_2 incubator. Then the overlay was carefully decanted, the monolayer was washed three times with MEM, and 0.2 ml of an anti-RS virus sheep hyperimmune serum (optimal dilution determined prior to use) plus 10 % complement were added.

Following incubation for 2 hr at 37 °C the supernatant was removed and the cells were stained with 0.3 % trypan blue solution. After careful washing with PBS, the foci were counted microscopically.

Animal experiments. Female BALB/c mice 16 to 20 gram (VEB Versuchstierproduktion, Schönwalde) were weighed daily throughout the experiment. The animals were anesthetized with hexobarbital i.p. and then infected with 100 µl virus suspension intranasally (tissue culture supernatant containing 10^4 PFU/ml of RSV). Norakin was administered 30 min before and 4 hr p.i. (each dose 0.5 mg in 200 µl distilled water) as an oral gavage. The controls were treated in the same manner with 0.15 mol/l NaCl solution.

Seven days post-infection the animals were killed by cervical dislocation. Hematoxylin/eosin-stained paraffin sections from lungs were examined histologically. The specificity of findings was checked by indirect immunofluorescence staining of frozen lung sections, using rabbit anti-RSV serum and homologous fluorescein-labelled anti-rabbit antibody to detect viral antigens.

Results

The antiviral effect of norakin against RSV replication in Vero cells was tested within the concentration range of 0.1—10 µg/ml (Fig. 1). Each point represents the average of three independent experiments where each compound concentration was tested in triplicate. The 50 % inhibitory concentration (ID_{50}) was determined graphically to be 1 µg/ml, 90 % inhibition being observed at 5 µg/ml. Toxic effects set in beyond the antiviral concentration range (Fig. 2). Cell division was completely blocked at 50 µg/ml. The cytostatic ID_{50} was determined graphically to be (30 ± 6) µg/ml, resulting in a therapeutic index *in vitro* of 30.

BALB/c mice infected with RSV develop lung lesions which can be used to evaluate the therapeutic efficacy of the tested compounds. Two doses of norakin (a total of 50 mg per kg) administered half an hour before and 4 hr

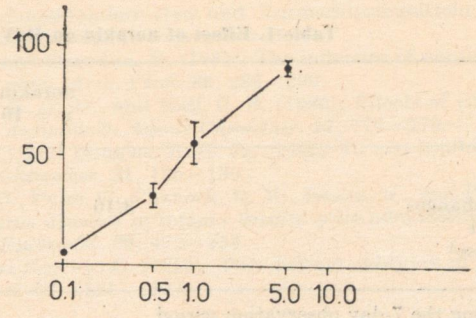

Fig. 1.
Influence of norakin on respiratory syncytial virus focus formation in Vero cells
Abscissa: norakin (µg/ml); ordinate: % focus concentration.

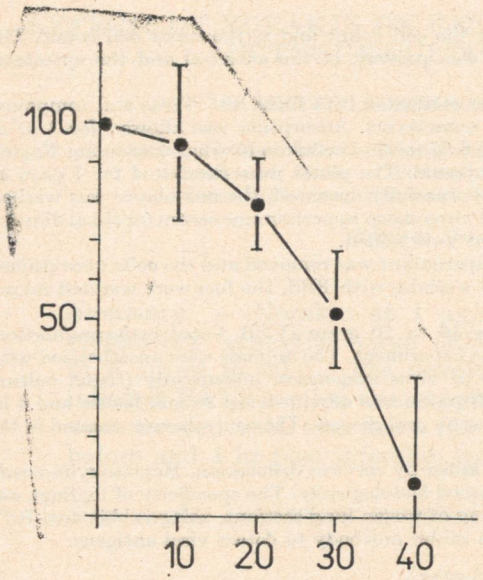

Fig. 2.
Effect of norakin on the division of Vero cells
Abscissa: norakin (μg/ml); ordinate: denotes the percentage of cell division as compared to untreated control cultures (in %).

p.i. suppressed the development of lung lesions in 70 % of the animals (Table 1).

Discussion

Respiratory syncytial virus is an important cause of infant morbidity and mortality posing great difficulties for vaccine development (Kim *et al.*, 1969; Parrot *et al.*, 1975). Recently, ribavirin has been shown to inhibit the replication of RSV in cell culture (Hruska *et al.*, 1980) and in cotton rats (Hruska *et al.*, 1982); it has been approved for aerosol treatment of RSV infections in infants (Hall *et al.*, 1983). Without doubt, therefore, antiviral chemotherapy is a way of controlling this disease.

We have tested norakin, a compound previously shown to be active against influenza A and B (Presber *et al.*, 1984; Heider *et al.*, 1985) and measles virus replication *in vitro*, and against influenza A and Sindbis virus (Veckenstedt

Table 1. Effect of norakin on RSV-infected BALB/c mice

	norakin n = 10		control n = 10	
		%		%
lung changes	3/10	30	10/10	100
weight increase[1]		8.2		4.7

[1] during the 7-day observation period

et al., 1985; Heider, 1986) in animals for its effect on RSV. Its mode of action as an inhibitor of influenza virus replication *in vitro* has been partially elucidated in that it inhibits virus penetration and interacts with haemagglutinin (Schroeder *et al.*, 1985; Ghendon *et al.*, 1986). RSV has proved to be as sensitive to norakin as influenza A virus and more sensitive than measles virus.

In two influenza virus animal models, the A/Victoria/3/75 (H3N2) — infected BALB/c mouse and in chicken infected with the strain A/gull/Potsdam/79 (H7N7), norakin was somewhat inferior to rimantadine treatment (Heider, 1986). At a total dose of 40 mg/kg (also in the two-dose treatment scheme as used for RSV) 100 % of the treated mice survived, compared to 50 % of controls, but there was only a 30 % reduction of lung lesions as compared to untreated mice. The effective *in vivo* doses of norakin against RSV and influenza appear to be similar.

In Sindbis virus-infected mice, norakin exhibits a certain therapeutic efficacy despite its only marginal anti-Sindbis virus activity *in vitro*. Optimal therapeutic effect was achieved in a treatment regimen of 10 mg/kg administered daily for 5 days (50 % survivors compared to zero in the controls). At higher norakin doses (25 and 50 mg/kg per day) the numbers of survivors decreased, while uninfected animals receiving the same norakin treatment showed no pathological signs (Veckenstedt *et al.*, 1985).

Optimal therapeutic schemes for norakin in influenza and RS virus-infected animals have yet to be determined. On the basis of molecular genetic analyses of sensitive and resistant influenza strains we are hoping to develop more potent and less toxic derivatives of norakin. In the case of RSV it is necessary to identify the molecular target of norakin to consider whether further investigation would be worth while.

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