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Effect of the virostatic norakin® (triperiden) on influenza virus activities

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Abstract

The effect of the virostatic norakin on various in vitro activities of influenza viruses was studied. The infectivity of the [A/PR/8/34 (H1N1)] strain for MDCK (Madin Darby canine kidney) cells was reduced by a factor of 10 with 10^{-7} M norakin. At 10^{-5} M, it was below 1% of the control value without norakin. At higher concentrations ($\geq 10^{-4}$ M), cytotoxic effects occurred. Neither hemolysis nor hemagglutination were affected by norakin concentrations up to 10^{-4} M. An in vitro fluorescence dequenching assay was used to study the viral fusion activity in the presence and absence of norakin. Fusion between influenza viruses and virus receptor-containing liposomes was not significantly affected up to norakin concentrations of 10^{-3} M. However, the intracellular pH in MDCK cells was raised from pH 5.3 (without norakin) to about pH 6 with 10^{-5} M norakin. This parallels the pH dependence of PR8 viral activities like hemolysis and fusion. We therefore suggest that norakin does not interact directly with the viral hemagglutinin, but inhibits viral infection through increase of the internal pH in the prelysosomal compartment.

Key words: Influenza virus; Hemagglutinin; Norakin (triperiden)

Presber et al. (1984) first demonstrated that norakin, an antiparkinsonism drug like amantadine, inhibits influenza virus infectivity. The inhibitory effect is only found if norakin is present before infection, indicating that an early step in the infection cycle is affected. Studies on the elucidation of the mechanism of norakin action have focused on the hemagglutinin (HA) molecule. Prösch et al. (1988, 1990)

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described mutations in the HA molecule in norakin-resistant viruses. In addition, Ghendon et al. (1986) showed a direct interaction of norakin with HA and suggested that norakin directly inhibits the conformational change of HA at acidic pH – a prerequisite for fusion. Unfortunately, only one concentration of norakin in the millimolar range was used in this study. Similarly, Schroeder et al. (1985) described a 2-fold decrease in hemolysis of human erythrocytes with norakin concentrations above 2×10^{-4} M when a low virus to cell ratio was used. We decided to study the influence of norakin over a wide concentration range on various HA-mediated viral activities, to test the hypothesis of direct norakin action on the hemagglutinin molecule. In a first step, we tested whether the virus strain used in our laboratory proved sensitive to norakin. For this purpose, the infectivity of PR8 [A/PR/8/34 (H1N1)] viruses on MDCK (Madin Darby canine kidney) cells was determined in the presence and absence of norakin. Results are presented in Fig. 1, where a reduction in infectivity can already be seen with 10^{-7} M norakin. As a control, the cytotoxicity of norakin in uninfected cells was tested on confluent monolayers of MDCK cells under normal culturing conditions. In the presence of 10^{-3} M norakin the cells were detached within 1 h, and at 10^{-4} M the same effect was noticed after 24 to 48 h. At lower concentrations, no cytotoxic effect was seen for up to 5 days. The solvent control (0.8% dimethylsulfoxide) showed no effect on cells during the observation period.

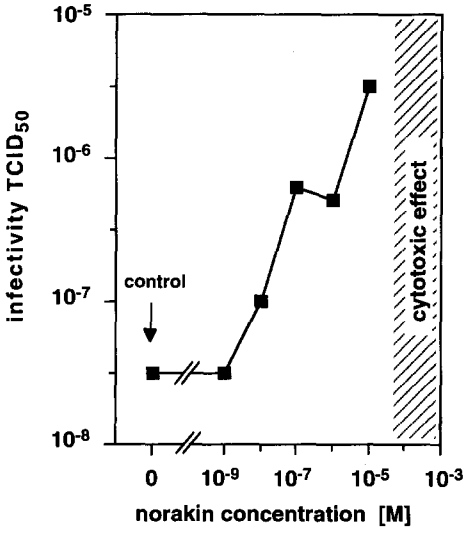

Fig. 1. Influence of norakin on the infectivity of PR8 influenza virus. The infectivity – expressed as $TCID_{50}$, Tissue Culture Infective Dose (Reed and Muench, 1938) – of PR8 influenza viruses was determined in MDCK cells as described (Wunderli et al., 1990). Norakin was added to the cells 15 min prior to infection at 37°C and was present at the indicated concentrations throughout the experiment. Virus production was monitored 3 days after infection as HA units determined in the supernatant. Furthermore, the cytotoxicity of norakin in MDCK cells was tested over the same concentration range as used in the infectivity test. Data are from 2 to 4 independent experiments.

Fusion of PR8 virus with small unilamellar liposomes containing the virus receptor G_{D1a} was monitored with the R18 dequenching assay (Hoekstra et al., 1984; Wunderli and Ott, 1990). Detergent dialysis liposomes (lipid composition: egg yolk phosphatidylcholine, phosphatidic acid, cholesterol, ganglioside G_{D1a} and R18 at molar ratios of 0.57/0.07/0.21/0.06/0.09) and virus particles were mixed in a cuvette containing phosphate-buffered saline (PBS: 10 mM Na_2HPO_4 and KH_2PO_4, 130 mM NaCl, pH 5.3, prewarmed to 37°C). The increase in fluorescence was recorded with a Perkin Elmer spectrofluorometer (slit width 10 nm, excitation 445 nm, emission 485 nm). The concentrations of liposomes and viruses (see legends) in the assay were chosen such as to obtain only 50% of the possible dequenching (data not shown) in order to avoid working with excess virus. Dequenching curves were found to follow second order kinetics (Wunderli and Ott, 1990) and thus can be described by DQ_{max} (the maximal dequenching reached) and t^* (the time to reach half-maximal dequenching). Norakin was added to the liposomes 15 min prior to virus addition at the indicated concentrations. As no effect was seen with this experimental arrangement, virus particles were additionally preincubated at 37°C in PBS (pH 7.4) with the indicated concentrations of norakin 15 min prior to starting the reaction by addition of liposomes. As summarised in Table 1, no significant effect of norakin was observed also with this experimental set-up at concentrations up to 10^{-4} M norakin and only a small change in t^* was seen at 10^{-3} M norakin. For comparison, amantadine was also tested in this in vitro fusion assay; no effect was observed at a concentration of 10^{-4} M.

The influence of norakin on the hemolytic activity of PR8 virus was studied at pH 5.0 using 2% human erythrocytes according to Yewdell et al. (1983). The half-maximal effect in the absence of norakin was reached at a virus concentration of 27.5 ± 7.5 μg viral protein per ml ($n = 2$). Norakin did not affect hemolysis: at concentrations between 10^{-8} M and 10^{-3} M half-maximal hemolysis occurred between

Table 1
Influence of norakin on the fusion kinetics of PR8 influenza viruses with G_{D1a}-containing liposomes

Norakin concentration [M]	DQ_{max}	t^* [min]
0	0.427 ± 0.005	2.42 ± 0.14
10^{-9}	0.375 ± 0.006	2.71 ± 0.23
10^{-8}	0.446 ± 0.006	2.40 ± 0.18
10^{-7}	0.389 ± 0.006	2.52 ± 0.22
10^{-6}	0.403 ± 0.007	2.62 ± 0.25
10^{-5}	0.408 ± 0.007	2.47 ± 0.21
10^{-4}	0.414 ± 0.008	2.71 ± 0.28
10^{-3}	0.484 ± 0.014	3.39 ± 0.47

Incubations of PR8 viruses (1 μg protein per ml) with R18-labelled liposomes (200 ng lipid per ml) at pH 5.3 (37°C) were performed in the presence of various concentrations of norakin as indicated. Viruses were preincubated with norakin for 15 min at 37°C before starting the fusion experiment. The respective norakin concentrations were kept constant throughout the experiment. Dequenching curves were analysed with the non-linear curve fitting program, proFit (Wunderli et al., 1993). Fitted curve parameters, DQ_{max} and t^*, are listed.

22 and 34 μg viral protein per ml. The hemagglutinating activity of PR8 viruses was tested as described by Fazekas de St. Groth and Webster (1966) using chicken erythrocytes. No effect on hemagglutination was detected at norakin concentrations up to 10^{-5} M. At 10^{-4} M norakin, hemagglutination was reduced by $10 \pm 8\%$ ($n = 7$) as compared to the control, and at 10^{-3} M norakin the reduction was about $42 \pm 7\%$ ($n = 7$).

Thus, although virus infectivity was reduced with norakin concentrations in the micromolar range, other virus activities, such as hemagglutination, hemolysis and fusion, were only affected at concentrations $\geq 10^{-4}$ M. At these concentrations, however, non-specific inhibitory effects of norakin on HA have been described. For instance, R18 inhibits viral fusion when incorporated into viruses at a molar ratio of 1:1 with HA (Wunderli et al., 1993).

It has to be noted that norakin is entrapped intracellularly in the prelysosome. For amantadine, another weak base, it has been shown (Richman et al., 1981) that its concentration is about 100 times larger in the prelysosome than outside the cell. The accumulation of weak bases in the prelysosomes leads to an elevation of pH in this compartment, and thus inhibition of the acid-dependent, HA-mediated fusion (Skehel et al., 1982). The same mechanism works with other amines like ammonium chloride, and also high concentrations of amantadine seem to exert their antiviral activity by raising the prelysosomal pH (Hay et al., 1985).

In a final set of experiments we therefore tested the hypothesis that norakin inhibits influenza infectivity by an increase in prelysosomal pH. MDCK cells were grown in Petriperm® culture plates, which have a flexible bottom that can easily be cut. After cells reached confluency, FITC-Dextran (fluorescein isothiocyanate-coupled dextran MW 40000, Sigma) was added to the culture medium (final concentration 2 mg/ml) in order to load prelysosomes with the pH-sensitive fluorescent probe. FITC-containing medium was removed after 48 h and an area of 3×4 cm of the cell layer was cut out, rolled up and inserted as a cylinder in a fluorescence cuvette ($1 \times 1 \times 4.5$ cm) containing EBSS (Earle's Balanced Salt Solution, Gibco) pH 7.4. As a control, a second cuvette with cells that were not treated with FITC was used in order to measure background fluorescence andior light scattering produced by the membrane with the cell monolayer. Samples were thermostated at 37°C in the Perkin Elmer spectrofluorometer (slit width 5 nm; emission 520 nm, excitation 450 and 490 nm, respectively). Under these conditions, the monolayers could be used for 1 to 1.5 h. Adsorbed FITC-Dextran was removed by changing the EBSS buffer until a stable signal was obtained (4 to 6 times). pH values were calculated from the F_{490}/F_{450} ratios relating them to a standard curve with 0.25 μg/ml FITC-Dextran in EBSS at pH values between 3 and 8 (Tycko et al., 1983).

We tested the influence of norakin and amantadine at various concentrations. Drug was added to the cuvette and changes in pH were recorded after 2 min. Fig. 2 shows the concentration-dependent increase in the prelysosomal pH. No significant increase in pH was noted at 4×10^{-9} M norakin as compared to the norakin-free control. At 4×10^{-8} M the pH tended to increase although the standard deviation at this concentration was large. At norakin concentrations $> 10^{-7}$ M the pH increased to values above pH 5.6. No significant difference was found between the

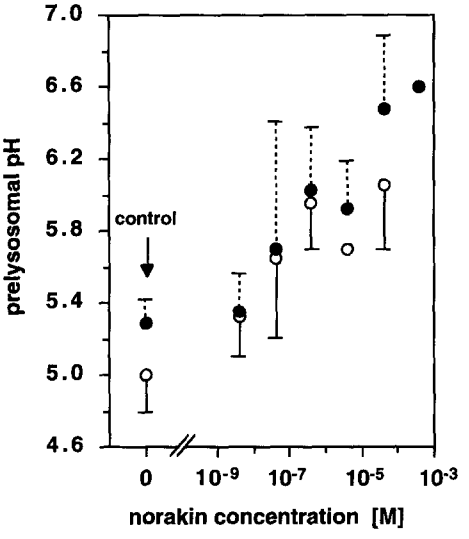

Fig. 2. Determination of the prelysosomal pH in MDCK cells. The fluorescence of MDCK cells, loaded with FITC-dextran, was measured both before and 2 min after the addition of norakin (●) or amantadine (○). The change in pH was calculated from the ratios of fluorescence at excitations 450 and 490 nm as described. Data are mean values (±S.D.) from 4 (norakin) or 2 (amantadine) independent experiments, except for the highest norakin concentration, which was only tested once.

action of norakin and amantadine. The pH-shift could be reversed by washing out the substance (not shown). As a positive control, NH_4Cl (20 mM) was used which resulted in a pH raise to 6.7–7.0 (data not shown).

We have shown here that norakin reduces the infectivity of PR8 virus by a factor of 10 at a concentration of 10^{-7} M. At this concentration the prelysosomal pH rose to a value of 5.6. This pH change coincides with results from Yewdell et al. (1983), who showed that hemolysis with PR8 [A/PR/8/34 (H1N1)] virus is optimal at pH 5.4 and not detectable at pH values above 5.6. In the R18 fusion assay, we also found a large drop of the fusion activity of PR8 viruses with liposomes in this pH range (Ott and Wunderli, unpublished results). Thus, this non-specific mechanism of a pH shift in the prelysosome is likely to be the basis for the antiviral effect of norakin. This interpretation is corroborated by findings of Schroeder et al. (1985) that norakin resistance is associated with a shift of the pH optimum for hemolysis towards higher pH values. As mentioned above, Prösch et al. (1990) noted that the mutations seen in HA in norakin-resistant strains are similar to those in strains resistant to high concentrations of amantadine, which also points to a pH effect.

In summary, we could not find evidence that norakin exerts its antiviral effect by a direct interaction with influenza virus. Norakin seems to inhibit virus infection, in analogy to other weak bases, by increasing the prelysosomal pH.

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