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Inhibition of influenza A virus replication by a kanamycin derivative

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Summary

We studied the antiviral activity and the mechanism of action of a new antiviral agent and kanamycin derivative, 1-N-eicosanoyl-3″-N-trifluoroacetyl kanamycin A (ETKA), against influenza A virus. From yield reduction assays with VERO cells, ETKA showed a significant antiviral activity with negligible cytotoxic effect. In the presence of 20 μg/ml of ETKA at which VERO cell growth was not inhibited, virus titer was suppressed to 11.2% of control, and at 100 μg/ml virus production was suppressed to more than 99%. ETKA markedly inhibited viral protein synthesis when cells were pretreated with the drug before infection, but there was no inhibition when the drug was added 15 min post-infection. ETKA did not inhibit virus adsorption and penetration. Nor did it affect the activity of viral RNA polymerase in vitro. We found that the drug had a direct inactivating effect on influenza A virus under acidic conditions. These results suggest that ETKA exerts its antiviral action mainly in the early stage, prior to uncoating by direct inactivation of the virus due to the acidic environment of the endocytic vesicle. Aerosol treatment with the drug protected mice against a lethal influenza A virus infection.

Kanamycin derivative; Influenza virus; Lysosomal pH; Aerosol treatment

Introduction

Ghendon and Mikhailovskaya (1982) showed that kanamycin A sulfate has weak antiviral activity against influenza virus infection in tissue cultures. Some compounds

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with higher alkyl or acyl groups in a molecule also show antiviral activity (Hoffman et al., 1973). Based on these reports, kanamycin A derivatives having a higher acyl group at the N-1 position were synthesized and kanamycin A analogs, having a higher alkylcarbonyl group, were found to exhibit much more potent antiviral activity against influenza viruses than kanamycin A sulfate (Matsuda et al., 1985a–c). The mechanism(s) by which the kanamycin A analogs inhibit the replication of influenza viruses have not been elucidated. In this study we investigated the antiviral activity of 1-N-eicosanoyl-3″-N-trifluoroacetyl kanamycin A (ETKA) against influenza A virus infection, both in vitro and in vivo, as well as the mechanism of action of this compound.

Materials and Methods

Drug and chemicals

ETKA was obtained from Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan. It was initially solubilized in dimethyl sulfoxide up to a concentration of 5 mg/100 µl. L-[^{35}S]methionine (600 Ci/mmol), [2,8-^{3}H]ATP (31.7 Ci/mmol) and [5-^{3}H]uridine (30 Ci/mmol) were purchased from Amersham Laboratories, U.K. Fluorescence isothiocyanate-dextran (FD-40) and chloroquine (molecular weight 515.9) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.. Amantadine hydrochloride was kindly provided by Japan Ciba-Geigy Co., Ltd, Takarazuka, Japan.

Cells and virus

African green monkey kidney (VERO) cells were grown in Eagle's minimum essential medium (MEM) containing 7% calf serum. Cells were routinely passaged every 3 days. For experiments and assays, cells were plated in 35-mm diameter plastic tissue culture dishes. Madin and Darby canine kidney (MDCK) cells were used for a plaque assay. They were grown in MEM containing 7% calf serum and 10% tryptose phosphate broth. We used VERO cells except for a plaque assay because ETKA appeared to have moderate cytotoxicity for MDCK cells. Influenza A/PR/8/34 (H1N1) virus was used for all experiments. Virus was grown in the allantoic cavity of 11-day-old chick embryos at 36°C for 2 days. Infectious virus was titrated on MDCK cells. Hemagglutinin (HA) titration was performed as described previously (Maeno and Kilbourne, 1970).

Virus yield reduction assays

VERO cell monolayers were pretreated with the drug for 30 min and infected at a multiplicity of infection (MOI) of 10 PFU per cell. After 1-h virus adsorption at 36°C, cells were further incubated for 24 h in the presence of the drug. Supernatant virus was quantified by HA titer or a plaque assay in MDCK cells.

Analysis of protein synthesis

VERO cell monolayers were pretreated with the drug for 30 min and infected at an MOI of 30 PFU per cell. After a 1-h virus adsorption period, cells were washed and incubated at 36°C in MEM containing 100 μg/ml of the drug. At appropriate times after infection, culture medium was removed and replaced with [^{35}S]methionine (10 μCi/ml) MEM lacking unlabeled methionine. The radioactive polypeptides were examined by 8.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Laemmli, 1970; Maeno et al., 1979).

Purification of virion and assay of influenza virus RNA polymerase

After clarification of the allantoic fluid, influenza virus was pelleted at 60000 × *g* for 1 h and suspended in phosphate buffered saline (PBS). The virus suspension was layered on top of a 10 to 40% (w/v) linear sucrose density gradient in PBS and centrifuged in a Hitachi RSP 28 rotor for 1 h at 60000 × *g*. The visible virus band was collected, resuspended in PBS and pelleted at 60000 × *g* for 1 h in an RSP 28 rotor. The polymerase assay procedure was based on the McGeoch and Kitron method (1975). The reaction mixture contained 50 mM Tris-hydrochloride (pH 7.7), 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.1 M NaCl, 0.1% Nonidet P-40, 0.2 mM GTP, CTP, UTP and [^{3}H]ATP, 0.25 mM ApG as primer, and virus sample and drug or H_2O. Reaction mixtures were incubated at 30°C for 1 h. Samples (50 μl) were mixed with 10% TCA, 0.1% sodium pyrophosphate for 30 min at 4°C and assayed for TCA-insoluble ^{3}H by spotting on glass fiber disks (GF/C filter). The disks were washed in two changes of 10% TCA, 0.1% sodium pyrophosphate, and two rinses of ethyl alcohol. Disks were then dried and counted in xylene-based scintillation fluid in a liquid scintillation counter.

Preparation of radiolabeled influenza virus

MDCK cell monolayers were infected at an MOI of 10 PFU per cell. Following a 1-h adsorption period, the cells were incubated with maintenance medium for 2 h at 36°C. [5-^{3}H]uridine (10 μCi/ml) was added, and after a labeling period of 18–20 h, supernatant was centrifuged at 1000 × *g* for 10 min. Virus was pelleted by centrifugation for 1 h at 60000 × *g* in a Beckman SW27 rotor and purified as described above.

Fluorescence probe measurement of intralysosomal pH

The pH inside the lysosomes of VERO cells was determined essentially according to the methods of Ohkuma and Poole (1978). Briefly, fluorescein isothiocyanate-dextran (FD) was dissolved in 10 mM-phosphate or acetate buffer containing 50 mM NaCl at various pH values (4.0–7.5) to give 1 μg/ml, and the fluorescence at each pH was measured with a fluorescence spectrophotometer FP-770 (Japan Spectroscopic Co., Ltd). The excitation wavelength was 400 nm to 500 nm and emission was measured at 519 nm. The standard pH curve was prepared by estimating the ratio of

fluorescence measured with excitation at 495 nm to that with excitation at 450 nm (FD 495/450 ratio). VERO cell monolayers were incubated in MEM containing 2 mg/ml FD for 48 h after which FD was observed by fluorescence microscopy to be distributed in the cytoplasm in a pattern corresponding with that of the lysosomes. The cells were then rinsed with Hanks' balanced salt solution, scraped with disposable cell scraper and suspended in PBS, pH 7.2 at 30°C. The excitation spectra of the cell suspensions were measured and the lysosomal pH values were estimated from the standard curve.

pH treatment

Purified influenza virus in PBS, pH 7.2 was adjusted to final pH values of 8.0, 7.0, 6.5, 6.1, 5.8 and 5.6 with 20 μg/ml of ETKA, held at each pH for 20 min at 36°C, and adjusted back to pH 7.2 with MEM. Virus was quantified by a plaque assay in MDCK cells. Furthermore, the direct effect on pH 5.8 and pH 7.0 was examined with various concentrations of ETKA. Buffers used were 0.85% NaCl/20 mM sodium acetate in a range of pH 5.2–5.8, and 0.85% NaCl/10 mM sodium phosphate in a range of pH 6.1–8.0.

Infection of mice

Four- to 5-week-old ICR mice were inoculated intranasally with 3 median lethal doses (LD_{50}) of mouse-adapted influenza A virus under 2,2,2-tribromoethanol anesthesia. Then each group, consisting of 20 to 23 mice, was placed in a sealed plastic cage with a volume of 3.0 l and exposed to aerosols of the drug with a concentration of 1 mg/ml in sterile distilled water for 30 min. Aerosols were generated with Nissho nebulizers and compressor. Aerosol treatment was started the same day 4 h after infection and continued for 4 days twice a day for 30 min. Survival of mice in groups was estimated over a 14-day period. The significance of the survival curves was determined by the generalized Wilcoxon test.

Results

Antiviral activity of ETKA in vitro and in vivo

Antiviral activity of ETKA against influenza virus was assessed by yield reduction assays on monolayers of VERO cells. As shown in Fig. 1, the concentration of ETKA required to reduce virus production by 50% was less than 10 μg/ml. At 50 μg/ml of ETKA, virus production was inhibited by more than 98%. As to the cytotoxic effect of ETKA, we could not find significant morphological changes in cultures treated with even 200 μg/ml of the drug. ETKA did not inhibit protein synthesis in uninfected VERO cells at a dose of 200 μg/ml (Table 1).

We next examined the effect of ETKA in an animal model. As shown in Fig. 2, aerosol treatment with 1 mg/ml of ETKA significantly improved the survival of mice

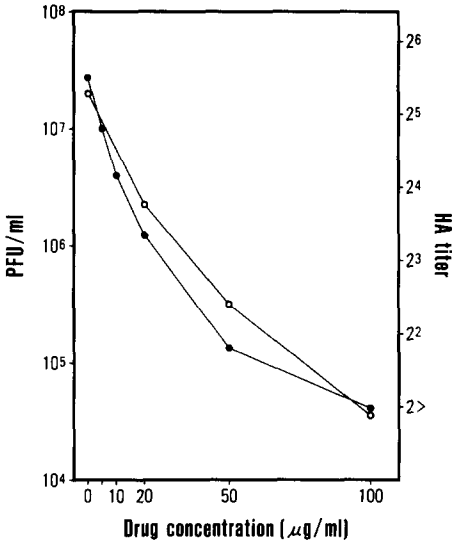

Fig. 1. Effect of ETKA on the replication of influenza virus. VERO cell monolayers were pretreated with the drug for 30 min and infected at an MOI of 10 PFU per cell. After 1 h virus adsorption at 36°C, cells were further incubated for 24 h in the presence of the drug. Supernatant virus was quantified by HA titer or by a plaque assay in MDCK cells. o—o, PFU/ml; ●—●: HA titer.

TABLE 1

Effect of ETKA on protein synthesis in uninfected VERO cells

Drug dose (μg/ml)	Counts of [^{35}S]methionine
0	624.9 ± 65.5 (100%)
100	663.3 ± 51.8 (106%)
200	651.3 ± 32.0 (104%)

Confluent monolayers of VERO cells were pulse-labeled with [^{35}S]methionine for 4–5 h with or without ETKA. Cells were removed with SDS, filtered, dried, and washed with 10% TCA and ethyl alcohol, and radioactivity was measured in liquid scintillation counter. Results are expressed in kilocounts per minute (kcpm). Values are means ± standard errors.

inoculated intranasally with 3 LD_{50} of virus ($P<0.05$).

Effect of ETKA on synthesis of viral and cellular proteins

We examined the time course of viral protein synthesis in the presence of ETKA at 100 μg/ml. Cells were pretreated with ETKA for 30 min before infection, infected with influenza virus at an MOI of 30 PFU per cell, incubated with maintenance medium containing ETKA, and labeled with [^{35}S]methionine hourly after 3 h post-infection (p.i.; Fig. 3). In the absence of ETKA, nucleoprotein (NP), membrane protein (M) and non-structural protein (NS) were clearly detected by 3–4 h p.i., while hemagglutinin (HA), neuraminidase (NA) and three P proteins (PB2, PB1, PA) by 5–

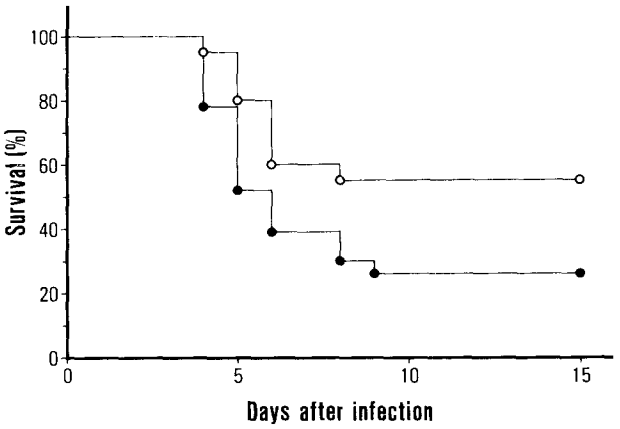

Fig. 2. Effect of ETKA treatment on the survival of influenza A virus-infected mice. Four-week-old ICR mice were inoculated intranasally with 3 LD_{50} of virus on day 0. Aerosol treatment with ETKA (1 mg/ml) was started on the same day and continued for 4 days twice a day. The numbers of mice per group were 23 in control group (●—●) and 20 in ETKA-treated group (○—○).

6 h p.i. In contrast, in the presence of ETKA at 100 μg/ml, the synthesis of viral protein was totally suppressed at 3 h after infection. When labeling with [^{35}S]methionine was done between 7–8 h, some NP polypeptide was detected. The inhibitory effect of ETKA on viral protein synthesis was noted only when VERO cells were pretreated with the drug. There was no inhibitory effect when the drug was removed before infection. Fig. 4 shows the inhibition of HA production following different times of addition of ETKA. Addition of ETKA (100 μg/ml) before infection completely inhibited HA production, but no inhibition was noted if the drug was added at 15 min (or later) p.i. This suggests that ETKA exerts an inhibitory action at an early stage of infection.

ETKA effect on early infection stages

We then studied the effect of ETKA on virus adsorption and/or penetration using radiolabeled influenza virus. ETKA increased rather than decreased the adsorption and/or penetration of influenza virus (Table 2).

We further examined the effect of ETKA on the activity of virus RNA polymerase. As shown in Fig. 5, ETKA showed 50% inhibition on virus RNA polymerase activity at concentrations of 200 μg/ml. ETKA had no apparent inhibitory effect at concentrations of less than 100 μg/ml.

ETKA effect on lysosomal pH

A pH-sensitive fluorescent probe was employed to measure the lysosomal pH of VERO cells held in PBS (pH 7.2), and the pH change was quantitatively determined by fluorescence spectrophotometer (Fig. 6). When 10 mM ammonium chloride was

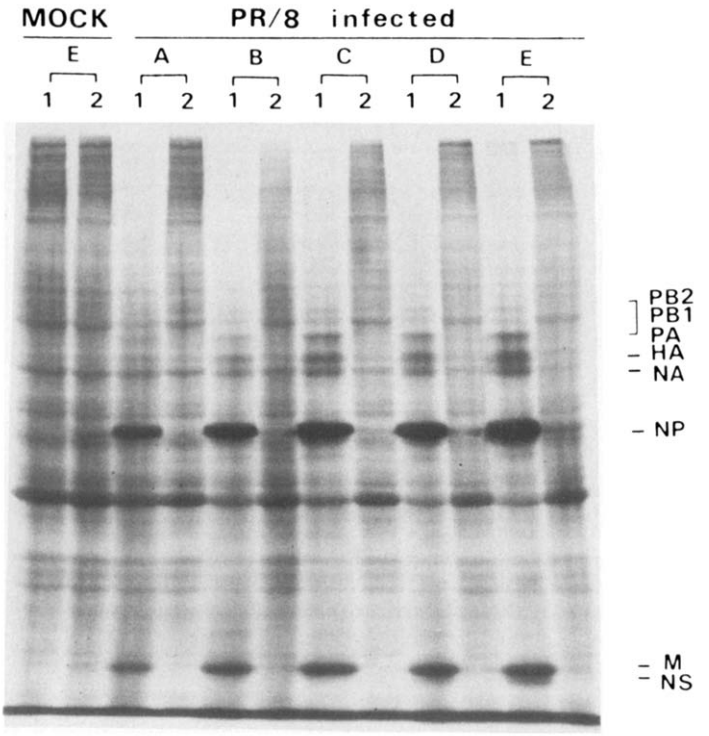

Fig. 3. Time course of viral protein synthesis in infected cells in the absence and presence of ETKA. VERO cell monolayers were pretreated with the drug for 30 min and infected or not at an MOI of 30 PFU per cell. One hour adsorption was followed by incubation at 36°C with maintenance medium in the absence or presence (100 μg/ml) of the drug. Labeling with [^{35}S] methionine (10 μCi/ml) was done between 3–4 h, 4–5 h, 5–6 h, 6–7 h, and 7–8 h postinfection. Lanes 1 and 2 indicate: 1, 0 μg/ml; 2, 100 μg/ml ETKA, respectively. A through E indicate: A, 3–4 h; B, 4–5 h; C, 5–6 h; D, 6–7 h; E, 7–8 h labeling, respectively.

added to the medium, the lysosomal pH rapidly increased to pH 6.3–6.4. When ETKA was added to the medium, the lysosomal pH increased by 0.1–0.2 pH unit (pH 5.9–6.0) over that of control. This is a weak effect when compared to that of other basic substances, such as amantadine or chloroquine. Kanamycin A sulfate did not affect the lysosomal pH at all (data not shown). These findings thus indicate that extracellular addition of ETKA causes only a slight increase of lysosomal pH.

Interaction between ETKA and influenza virus at low pH

We examined whether ETKA had any direct effect on the infectivity of influenza virus at various pH environments. Fig. 7 shows that ETKA had marked inactivation activity under acidic conditions (pH≤6.1) at a concentration of 20 μg/ml. In contrast, in the absence of ETKA, inactivation activity was not observed at a pH greater than 5.6. We further evaluated the concentration-response of ETKA at pH 5.8 and pH 7.0 (Fig. 8); at pH 5.8 it increased in a concentration-response manner. The infectivity of

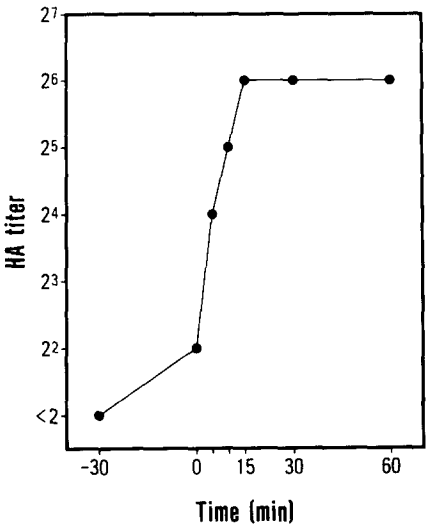

Fig. 4. Time dependence of ETKA inhibition. VERO cell monolayers were allowed to adsorb virus (10 PFU/cell) at 4°C for 30 min and were washed with PBS, after which cells were incubated at 36°C. ETKA (100 μg/ml) was added to the cultures at the indicated times before or after infection at 36°C. Cells and culture fluids were harvested 24 h after infection, frozen and thawed, and assayed for HA activity.

TABLE 2

Effect of ETKA on the adsorption and/or penetration of influenza virus

Drug dose (μg/ml)	Adsorption time (min)	Counts of [5-^{3}H]uridine
0	10	361.0 ± 16.0
	60	529.0 ± 8.0
100	10	904.5 ± 30.5
	60	1189.5 ± 25.5

VERO cell monolayers were pretreated with the drug for 30 min and infected with [5-^{3}H]uridine-labeled influenza virus in the presence of the drug. After 10 min and 60 min virus adsorption at 36°C, cells were washed with PBS, and removed with SDS, and cell-associated radioactivity was measured in a liquid scintillation counter. Results are expressed as counts per minute (cpm). Values are means ± standard errors.

influenza virus was reduced by more than 99.9% at a concentration of 20 μg/ml. At pH 7.0, however, ETKA had no inactivating activity. In addition, we found that the HA activity also was inhibited under acidic conditions. A direct inactivation at pH 5.8 was not observed by the addition of amantadine or chloroquine (data not shown).

Discussion

The present study showed that ETKA suppressed the synthesis of viral proteins without affecting host cellular protein synthesis. However, the addition of the drug after a 15 min adsorption period did not impair viral protein synthesis, suggesting that

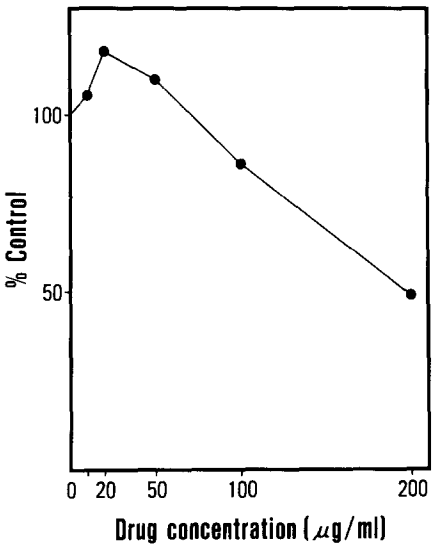

Fig. 5. Effect of ETKA on influenza virus RNA polymerase activity. The reaction mixture with virus sample and drug was incubated at 30°C for 1 h and the samples were withdrawn and assayed for TCA-insoluble ^{3}H. Results are expressed as a percentage of the values obtained in control mixture without any drug. One hundred percent of RNA polymerase activity was 38261 cpm.

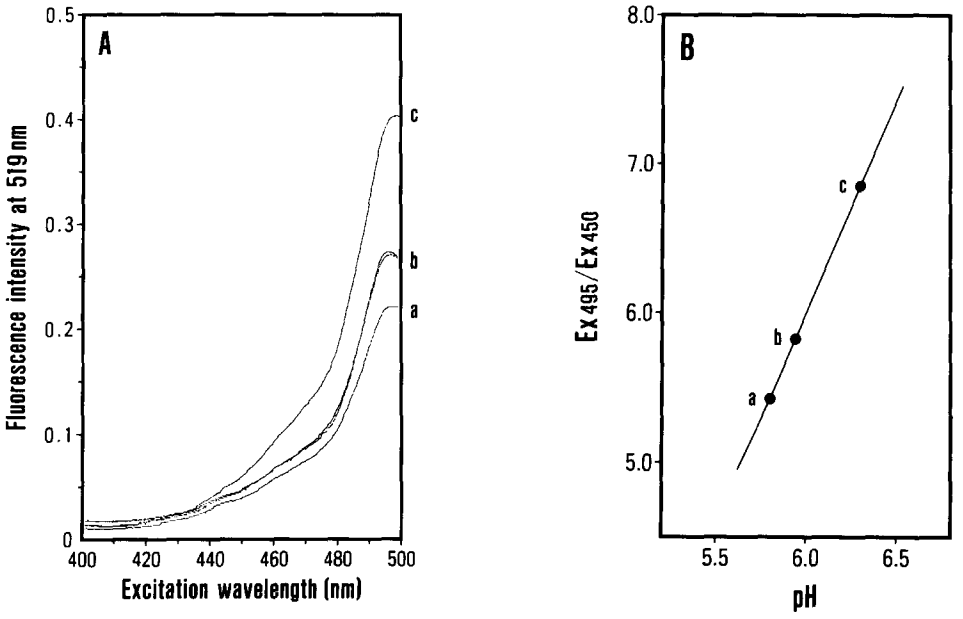

Fig. 6. Excitation spectra (A) and the lysosomal pH (B) of VERO cells containing fluorescein isothiocyanate-dextran in their lysosomes. The pH was calculated from the 495/450 ratio (fluorescence with excitation at 495 and 450 nm). a, control; b, ETKA (50 and 100 μg/ml); c, 10 mM ammonium chloride.

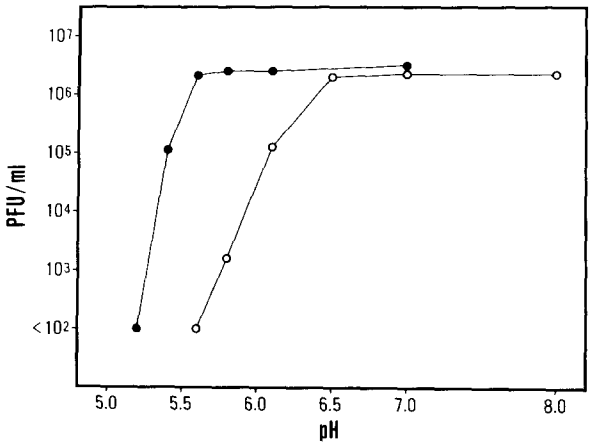

Fig. 7. Effect of ETKA on influenza virus infectivity at various pH treatments. Virus (in PBS, pH 7.2) was adjusted to final pH values of 8.0, 7.0, 6.5, 6.1, 5.8 and 5.6 with the drug concentrations of 20 μg/ml, held at each pH for 20 min, and adjusted again to pH 7.2 with MEM. Virus infectivity was quantified by a plaque assay in MDCK cells. ●—●: control; ○—○: ETKA.

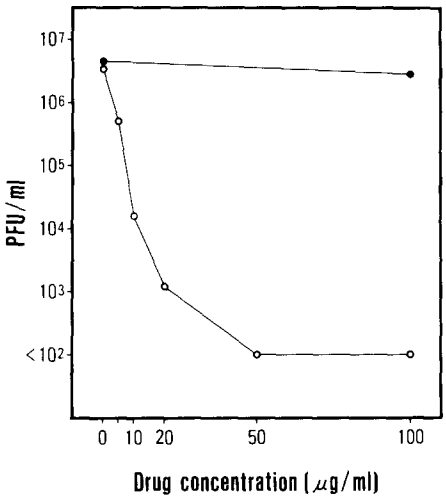

Fig. 8. Effect of ETKA concentration on influenza virus infectivity at pH 5.8 and pH 7.0. Virus (in PBS, pH 7.2) was adjusted to final pH values of 5.8 and 7.0 with various concentrations of ETKA, held at each pH for 20 min, and adjusted again to pH 7.2 with MEM. Virus infectivity was quantified by a plaque assay in MDCK cells. ●—●: pH 7.0; ○—○: pH 5.8.

it might act at an early stage of viral replication. ETKA had no inhibitory effect on the activity of influenza RNA polymerase in vitro, indicating that the compound may inhibit the replication of influenza A virus at a stage preceding the primary transcription.

ETKA increased the adsorption and/or penetration of ^{3}H-uridine-labeled influenza

virions. On the other hand, ETKA showed direct inactivating effect on influenza virus under mildly acidic conditions (≤pH 6.1). Aerosol treatment of influenza A virus infection in mice improved the survival. Additionally we found that the acute toxicity of ETKA in mice was very low, with a 50% lethal dose > 400 mg/kg [following a single intraperitoneal injection (unpublished data)].

The pH in the prelysosomal endocytic vesicles (endosomes or receptosomes) is low (Maxfield, 1982), and uncoating of influenza virus occurs in the endosomes before reaching the secondary lysosomes, mediated by the low pH-induced fusion between viral envelope and endosomal membrane (Marsh et al., 1983; Yoshimura and Ohnishi, 1984). Various weak basic substances such as ammonium chloride, amantadine and chloroquine exert their antiviral activities by increasing vesicle pH and preventing uncoating (Ohkuma and Poole, 1978; Shibata et al., 1983; Yoshimura and Ohnishi, 1984; Richman et al., 1986).

ETKA also has a similar effect on the pH in the vesicles of VERO cells, but the elevation of the vesicle pH following ETKA treatment is weaker than that of other basic antiviral substances. ETKA effects a direct inactivation of influenza virus under acidic conditions, an effect that is not observed with amantadine or chloroquine. It is suggested, therefore, that ETKA, incorporated with the virions, inactivates virions directly in the endosomal or lysosomal vesicles, although a slight increase in vesicle pH induced by ETKA may also be involved in the inhibitory action of this drug. It is also possible that ETKA attached to the membrane binds to viral RNA during uncoating and results in decreasing protein synthesis and virus yield. The lysosomal pH of MDCK cells in suspension has previously been reported to be pH 5.6 (Shibata et al., 1983). The pH inside the lysosomes of the VERO cells observed was slightly higher than that reported for MDCK cells, perhaps due to differences in cells or culture conditions.

The addition of an acyl group to kanamycin A makes it lipophilic and more readily incorporated into cells, and the introduction of trifluoroacetyl group at the N-3″ position reduces its cytotoxicity (Matsuda et al., 1986). In addition, N-higher acyl derivatives of other aminoglycosides also exhibit almost the same antiviral activity against influenza viruses (Matsuda et al., 1987). These findings indicate to some extent that N-acylation of kanamycin A with a higher acyl group is essential for antiviral activity. It is tempting to speculate, however, that the kanamycin A nucleus could be replaced with other aminoglycoside antibiotics without loss of antiviral activity. Direct inactivation of influenza virus under acidic conditions by ETKA appears to depend on the structure of the N-acyl group of kanamycin A. The exact mechanism by which ETKA inhibits influenza virus remains unresolved.

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