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Antiviral Research 26 (1995) 11–25

Antiviral Research

Mode of action of the anti-influenza virus activity of plant flavonoid, 5,7,4′-trihydroxy-8-methoxyflavone, from the roots of *Scutellaria baicalensis*

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Received 17 April 1994; accepted 6 September 1994

Abstract

When mouse-adapted influenza virus A/PR/8/34 (A/PR8) (10 PFU/cell) was adsorbed to Madin-Darby canine kidney (MDCK) cells at 4°C for 1 h and incubated at 37°C, release of the virus from the cells was detected in the medium from 4 h after incubation and reached to plateau at 8 h. However, 5,7,4′-trihydroxy-8-methoxyflavone (F36) from the roots of *Scutellaria baicalensis* significantly reduced this single-cycle replication of A/PR8 from 4 h to 12 h after incubation by dose-dependent manner and the dose which decrease the virus titer one tenth was 11 μM. F36 (50 μM) did not inhibit the adsorption of A/PR8 to MDCK cells, but reduced release of the virus in the medium, when it was added at 0 or 2 h after the incubation. The cell-associated virus determined by sialidase activity was also reduced by F36 treatment at 0 or 2 h. F36 also inhibited the fusion of A/PR8 with liposomes containing bovine brain mixed gangliosides at pH 5.0. However, F36 little affected on the elongation activity of the viral RNA-dependent RNA polymerase in vitro. These results suggest that F36 reduces the replication of A/PR8 by inhibiting the fusion of the virus with endosome/lysosome membrane which occurs at early stage of virus infection cycle. Whereas, when F36 was added to the MDCK cells infected with A/PR8 at 3 or 4 h after incubation, release of the virus in the medium was reduced but the cell-associated virus was increased in comparison with control. Scanning and transmission immunoelectron microscopic studies revealed that F36 inhibited the budding of progeny A/PR8 from the MDCK cell surface and microvilli, when it was added at 3 h after incubation. The accumulation of the A/PR8

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SSDI 0166-3542(94)00062-X

antigen was observed on the cell surface by immunofluorescence and transmission immunoelectron microscopies by the addition of F36. These results suggest that F36 also shows anti-influenza virus activity against A/PR8 by inhibiting the budding of the progeny virus from the cell surface, when it was added at budding stage of virus infection cycle.

Keywords: Flavonoid; Influenza virus; Sialidase; Budding; Fusion

1. Introduction

Influenza virus infection is initiated by the attachment of hemagglutinin to the sialic acid residue of glycoconjugate receptors on the host cell surface (Kingsbury, 1990). Then the virus is internalized and proceed through the different stages of the virus replication cycle, such as fusion of viral and endosome/lysosome membranes, and replication of viral RNA and proteins (Kingsbury, 1990). Finally, the progeny viruses are released by budding from the infected host cells (Kingsbury, 1990). The sialidase [neuraminidase, enzyme classification EC 3.2.1.18] catalyses cleavage of terminal sialic acid residues from the sialoglycoconjugate receptors (Gottschalk et al., 1972), and presumably aids in the elution of newly formed viruses from the infected host cells on budding stage (Murti and Webster, 1986). Therefore, influenza virus sialidase inhibitors may inhibit the virus infection.

Previously, we reported that 5,7,4′-trihydroxy-8-methoxyflavone (F36) isolated from the roots of *Scutellaria baicalensis* was shown to have a specific inhibitory activity against influenza virus sialidase but not mouse liver sialidase (Nagai et al., 1989; Nagai et al., 1990), and that this flavone also showed anti-influenza virus activity in Madin-Darby canine kidney (MDCK) cells, in the allantoic sac of embryonated egg and in vivo using BALB/c mice (Nagai et al., 1990; Nagai et al., 1992). Recently, new analogs of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en), 4-guanidino-Neu5Ac2en and 4-amino-Neu5Ac2en, were synthesized and shown to have specific inhibitory activity against influenza virus sialidase (von Itzstein et al., 1993). These compounds also showed anti-influenza virus activity not only in MDCK cells but also in vivo using mice and ferrets (von Itzstein et al., 1993). But the mechanism of action of anti-influenza virus activity of the sialidase inhibitors have never been studied.

The present paper describes the mechanism of action of in vitro anti-influenza virus activity of F36 possessing inhibitory activity for influenza virus sialidase.

2. Materials and methods

2.1. Materials

F36 was purified from the root of *S. baicalensis* (Tomimori et al., 1982) or synthesized according to the previously described procedures (Morita, 1960; Iinuma et al., 1984). Sodium *p*-nitrophenyl-*N*-acetyl-α-D-neuraminate (PNP-NeuAc) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dipalmitoylphos-

phatidylcholine (DPPC), cholesterol, dicetylphosphate, ganglioside (Type III from bovine brain) and sodium cholate, used for liposome preparation, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Octadecyl rhodamine B-HCl (R 18), used for labeling of influenza virus, was purchased from Molecular Probes Inc. (Eugene, OR, USA). Adenylyl(3′ → 5′)guanosine (ApG) was obtained from Sigma, and RNase inhibitor (from human placenta) was from Wako. [2,8-^{3}H]ATP (1.0 TBq/mmol) was purchased from DuPont/NEN Research Products (Wilmington, DE, USA).

2.2. Cells and viruses

MDCK cells were grown in Eagle's minimum essential medium (EMEM) containing 10% inactivated fetal bovine serum (FBS), penicillin G (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) (growth medium). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Influenza virus A/PR/8/34 (mouse-adapted) was obtained from the Kitasato Institute (Tokyo, Japan). The virus was grown in the allantoic cavity of 10-day-old embryonated hen eggs at 34°C for 2 days. The allantoic fluid was harvested and clarified at 1000 × g for 20 min, and then resulting supernatant fractions were stored in small portions at −80°C until use.

2.3. Purification of influenza virus

After clarification of the allantoic fluid, influenza virus was pelleted at 80 000 × g for 45 min and suspended in phosphate-buffered saline (PBS), pH 7.4. The virus suspension was held at 4°C overnight, layered on the top of a 30 to 60% (w/v) linear sucrose density gradient in PBS and centrifuged in a Beckman SW41Ti rotor at 80 000 × g for 2 h. The sialidase active fractions were collected, diluted with PBS and pelleted at 80 000 × g for 2 h.

2.4. Sialidase assay

Influenza virus sialidase activity was assayed as previously described (Nagai et al., 1990). In brief, the reaction mixture containing 25 nmol of PNP-NeuAc and influenza virus as the enzyme in 25 mM citrate-phosphate buffer, pH 6.5, was incubated at 37°C for 15 min in microtiter plate, and the p-nitrophenol liberated was determined from the absorbance at 405 nm with a Microplate Reader Model 450 (Bio-Rad Laboratories). MDCK cell-associated influenza virus sialidase activity was assayed as follows. The virus-infected cell monolayers in the well of tissue culture plate were washed with PBS, and 200 μl of PBS containing 50 nmol of PNP-NeuAc was added to the well. The culture plate was incubated at 37°C for 15 min, and the p-nitrophenol in the supernatant was determined from the absorbance.

2.5. Preparation of liposome

Unilamellar liposomes were prepared by a detergent-dialysis method (Zumbühl and Weder, 1981), described by Yamazaki et al. (1992). DPPC, cholesterol, dicetylphos-

phate, and bovine brain mixed ganglioside (35:45:5:15 as molar ratio) were dissolved in chloroform/methanol (1:1, v/v), and dried under a stream of N_2 then overnight in vacuo. The dried lipids were dispersed at 15 mg/ml in a 20 mM sodium borate buffer, pH 8.4, containing 0.14 M NaCl and sodium cholate (lipids/detergent = 1:0.6 as molar ratio). The mixed-micelle dispersion was vortexed and sonicated in an ultrasonic bath. The mixed-micelle suspension was dialyzed against the 20 mM sodium borate buffer, pH 8.4, containing 0.14 M NaCl and then PBS, pH 7.5, at room temperature in an Ultrafiltration apparatus (Amicon) equipped with a YM5 disc membrane under a N_2 atmosphere. The liposome suspension obtained was stored at 4°C.

2.6. Fusion assay

F36 was dissolved in 50% dimethyl sulfoxide (DMSO). The fusion activity of influenza virus was assayed as described previously (Hoekstra et al., 1984; Lüscher-Mattli and Glück, 1990). Ethanol solution (6 μl) of 2 mM octadecyl rhodamine B-HCl (R 18) was rapidly injected into 1 ml of purified virus suspension (0.75 mg/ml as protein), and incubated at room temperature for 1 h in the dark. After the incubation, R 18-labeled virus preparation was washed with PBS, pH 7.5, by centrifugation at 15000 rpm for 15 min to remove excess R 18. The R 18-labeled virus preparation (18 μl, 2.6×10^7 PFU) was incubated with the liposome suspension (240 μl) and F36 solution (10 μl) at 4°C for 30 min. After this prebinding period, the reaction mixture was diluted with PBS, pH 7.5, to a final volume of 3 ml at 37°C. The fluorescence was measured at 37°C by excitation at 560 nm and emission at 590 nm. After measurement of the initial fluorescence at pH 7.5 (F_0), the pH was adjusted to 5.0 by addition of 0.25 M acetic acid (114 μl). The increase of fluorescence (F), resulting from the fusion of liposomal and viral membranes at pH 5.0, was monitored for 10 min. Then, Triton X-100 (60 μl, final 0.2% v/v) was added to obtain the fluorescence at 100% dequenching (F_t). The percentage of fluorescence-dequenching (FDQ) was calculated as follows:

$$\%\mathrm{FDQ} = \left[(F - F_0)/(F_t - F_0)\right] \times 100$$

2.7. RNA-dependent RNA polymerase assay

F36 was dissolved in 50% DMSO. Influenza virus RNA polymerase activity was assayed as previously described (McGeoch and Kitron, 1975). The reaction mixture (0.11 ml) containing 50 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.1 M NaCl, 0.1% Nonidet P-40, 0.25 mM ApG, RNase inhibitor (220 units/ml), 0.2 mM GTP, 0.2 mM CTP, 0.2 mM UTP, 0.2 mM [^{3}H]ATP (1.1 MBq/μmol), purified virus (0.6 mg/ml as protein) as the enzyme and F36 solution (10 μl) was incubated at 30°C for 1 h. The produced RNA was precipitated by the addition of 0.1% bovine serum albumin (BSA) (0.25 ml) and mixture of 10% trichloroacetic acid (TCA) and 0.1% sodium pyrophosphate (1 ml) for 30 min in ice bath. The TCA-insoluble material was spotted on the glass fiber disc (GF/C filter, Whatman) by filtration. The disc was washed with the mixture of 10% TCA and 0.1% sodium pyrophosphate then ethanol, and dried in air. TCA-insoluble ^{3}H on the disc was counted in toluene-based scintillation fluid with liquid scintillation counter (Aloka).

2.8. *In vitro anti-influenza virus experiments*

F36 was dissolved in methanol. For the assay, MDCK cells were plated in 48 wells plastic tissue culture plates (Costar; 11.3 mm well diameter). Confluent monolayers of the cell cultures were adsorbed with mouse-adapted influenza virus A/PR/8/34 at a multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell (approx. 1.1×10^6 PFU) in 100 μl of PBS containing 1% BSA at 4°C for 1 h. After the adsorption, the cells were washed with PBS and incubated at 37°C for 10–12 h under 5% CO_2 atmosphere in 0.5 ml of EMEM containing 1% BSA, acetyltrypsin (3 μg/ml) and antibiotics (maintenance medium). The cells were incubated in the absence or presence of F36 solution (5 μl), which was added to the well of a culture plate at appropriate time. The infectious virus titer in the medium was determined by infectivity to MDCK cells ($TCID_{50}$). The monolayers in the culture plate were separated from the medium, washed with PBS to remove the dead cells resulting from infection of the virus, and the viable cells were determined by a colorimetric method which is based on the in situ reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells (Pauwels et al., 1988) as previously described (Nagai et al., 1992). The solvent control (final 1% methanol) showed no cytotoxic effects on the cells during the incubation.

2.9. *Immunofluorescence and electron microscopy*

For scanning electron microscopy (SEM) and cytoplasmic immunofluorescence, MDCK cells were grown on polyethyleneimine-treated coverslips (10-mm diameter) in 24 wells plastic tissue culture plates (Corning; 16-mm well diameter). Confluent monolayers of the cells were adsorbed with mouse-adapted influenza virus A/PR/8/34 (10 MOI, 200 μl) as cells were adsorbed with mouse-adapted influenza virus A/PR/8/34 (10 MOI, 200 μl) as described above. After the adsorption, the cells were washed with PBS and incubated in 1 ml of the maintenance medium at 37°C for 3 h under 5% CO_2 atmosphere. Then F36 solution (final 50 μM) was added to the well of culture plate, and the cell cultures were incubated for another 6 h in the dark. For SEM, the cell monolayers on the coverslips were washed with PBS, fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C, and post-fixed with 1% OsO_4 in PBS for 1 h at 4°C. After washing with PBS, the cultures were dried in *t*-buthanol by freeze-dry method with JFD-300 (JEOL). The cultures were mounted on stubs, sputtered platinum at 70 Å thickness with Sputter Coater, and observed by a JSM-6301F scanning electron microscope (JEOL). For the cytoplasmic immunofluorescence, the cell monolayers on the coverslips were washed with PBS and fixed in acetone for 30 min at 4°C. Indirect immunofluorescence staining was carried out by using mouse anti-whole influenza virus A/PR/8/34 antiserum and FITC-labeled rabbit anti-mouse IgG antiserum, then the sample was mounted in PermaFluor Aqueous Mounting Medium (Shandou/Lipshaw/Immunon). The immunofluorescence was examined with a transmission fluorescence microscope.

For transmission immunoelectron microscopy (TIEM), confluent monolayers of MDCK cell cultures in plastic tissue culture dishes (Corning; 35-mm diameter) were

adsorbed with mouse-adapted influenza virus A/PR/8/34 (100 μl) as described above. After the adsorption, the cultures were washed with PBS and incubated in 2.5 ml of maintenance medium at 37°C for 3 h under 5% CO_2 atmosphere. Then F36 solution (final 50 μM) was added to the dish, and the cell monolayers were incubated for further 6 h in the dark. MDCK cell monolayers were washed with PBS, and stained by using mouse anti-whole influenza virus A/PR/8/34 antiserum and peroxidase-labeled rabbit anti-mouse IgG antiserum. Cells were fixed overnight with 2.5% glutaraldehyde in PBS, post-fixed with 1% OsO_4 in PBS for 1 h, dehydrated with ethanol and embedded in Epon resin. After polymerization, the Epon blocks were broken off the plastic culture dishes, re-embedded from the reverse side in Epon resin, sectioned with Microtome. The ultra thin sections were placed on copper grids and stained with 2% uranyl acetate and Raynold's lead citrate and viewed with a JEM 1200 EXII electron microscope (JEOL).

3. Results

3.1. Effect of F36 on single-cycle replication of influenza virus in MDCK cells

The effects of F36 on single-cycle replication of mouse-adapted influenza virus A/PR/8/34 in MDCK cells were studied. When the influenza virus were adsorbed to MDCK cells at 4°C and then incubated at 37°C, infectious virus was increased in the medium from 4 h after the incubation and reached to plateau at 8 h (Fig. 1A). Whereas, when the influenza virus infected MDCK cells were incubated in the presence of F36 (50 μM), infectious virus titer was significantly reduced in the medium from 4 h to 12 h (Fig. 1A). When the anti-influenza virus activity of F36 on single-cycle replication was estimated at 10 h after incubation, 96% of cells survived in the presence of F36 (100 μM), although 16% of MDCK cells became extinct by the infection in the absence of

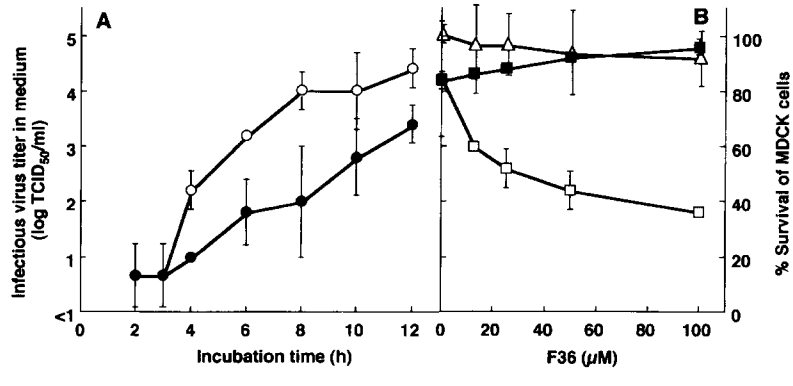


Fig. 1. Effect of F36 on single-cycle replication of influenza virus in MDCK cells. MDCK cell monolayers were adsorbed with mouse-adapted influenza virus A/PR/8/34 at a MOI of 10 PFU/cell for 1 h at 4°C, washed with PBS and added F36 solution. (A) The cells were incubated at 37°C for 12 h in the absence (●) or presence of F36 (50 μM) (○). (B) Viable cells were determined by MTT assay on infected (■) or mock-infected (△) conditions, and the infectious virus titer in the supernatant was evaluated by $TCID_{50}$ (□) at 10 h after incubation. Values represent mean ± S.D. ($N = 3$).

Table 1
Time-dependent effects of addition and removal of F36 on single-cycle replication of mouse-adapted influenza virus A/PR/8/34 in MDCK cells

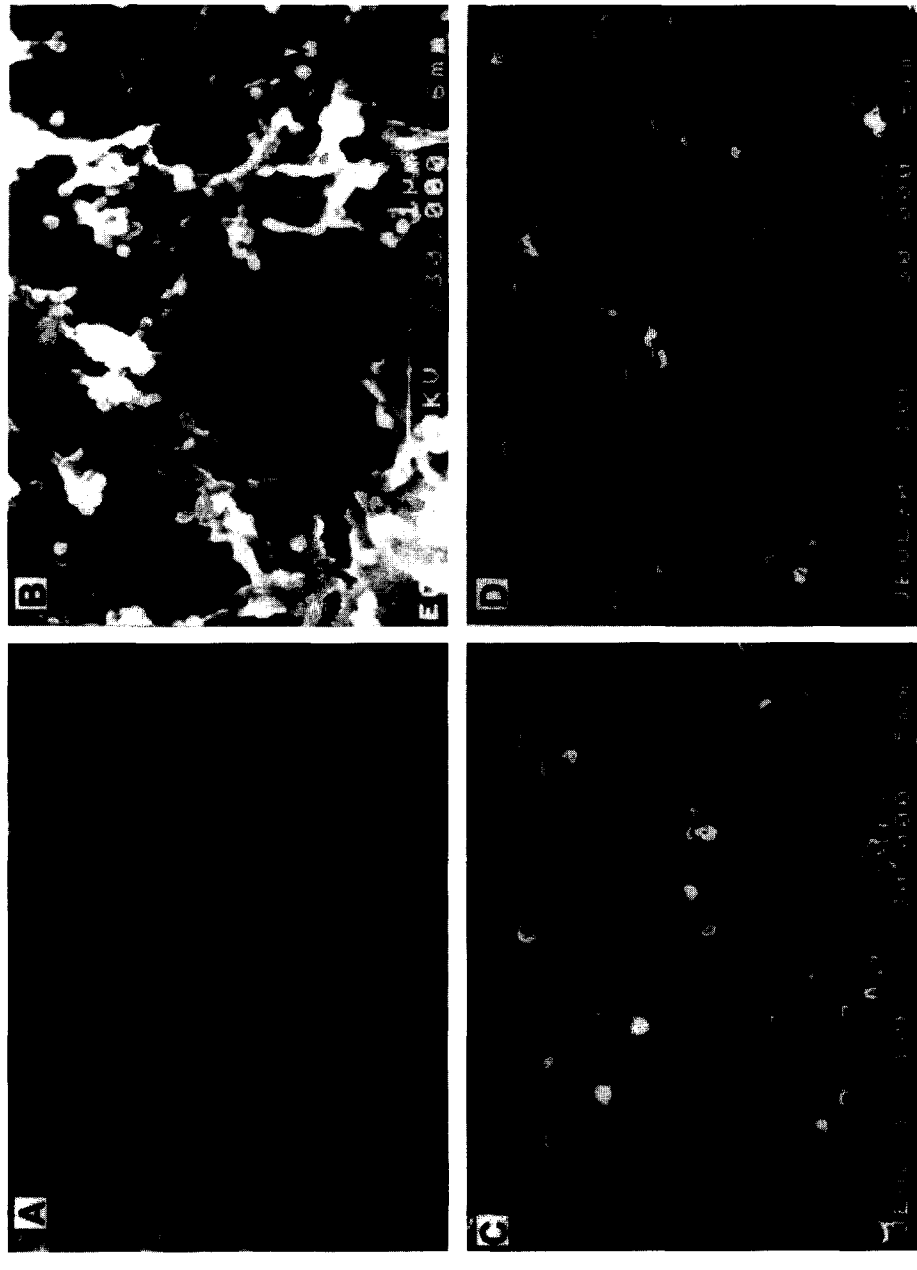
Duration of F36 treatment (h)	Infectious virus in medium (log $TCID_{50}$ /ml) [a]	Cell-associated virus (units/well as sialidase) [a,b]
Treatment on adsorption period [c]		
$-1 \rightarrow 0$	4.4 ± 0.5	n.d.
Post-treatment [d]		
$+0 \rightarrow +10$	3.6 ± 0.3	0.020 ± 0.008
$+2 \rightarrow +10$	4.0 ± 0.0	0.036 ± 0.025
$+3 \rightarrow +10$	4.0 ± 0.3	0.303 ± 0.048
$+4 \rightarrow +10$	4.2 ± 0.3	0.238 ± 0.033
$+6 \rightarrow +10$	4.6 ± 0.0	0.185 ± 0.002
Treatment [e]		
$+0 \rightarrow +2$	4.0 ± 0.0	n.d.
$+0 \rightarrow +3$	4.0 ± 0.0	n.d.
$+3 \rightarrow +6$	4.0 ± 0.9	n.d.
Control [f]	5.0 ± 0.3	0.176 ± 0.005

[a] Values represent mean ± S.D. of 3 experiments.
[b] One unit was defined as the amount of enzyme which hydrolyzed 1 μmol of PNP-NeuAc/min.
[c] MDCK cells were adsorbed by influenza virus (10 MOI) at 4°C for 1 h in the presence of F36 (50 μM), then washed unadsorbed virus and F36, and incubated at 37°C for 10 h.
[d] The cells were adsorbed by the virus (10 MOI) at 4°C for 1 h, and then incubated at 37°C for 10 h in the absence or presence of F36. F36 (50 μM) was added during the indicated time.
[e] The cells were adsorbed by the virus at 4°C for 1 h, and F36 was added during the indicated time. Then cells were washed and incubated in drug-free maintenance medium until 10 h.
[f] Virus-infected and 1% methanol (final concentration) but not F36-treated cells.
n.d. = not determined.

the flavone. Dose-dependent reduction of infectious virus titer was observed in the medium, and the dose of F36 which decrease the virus titer one tenth was 11 μM (Fig. 1B). Amantadine showed no anti-influenza virus activity in the same condition of Fig. 1B (data not shown). F36 showed little effect on the viability of MDCK cells at 12.5–100 μM by MTT assay (Fig. 1B).

3.2. Time-dependent effects of addition or removal of F36 on influenza virus replication in MDCK cells

The time-dependent effects of F36 were examined on the single-cycle replication of mouse-adapted influenza virus A/PR/8/34 in MDCK cells. When MDCK cells were adsorbed by the virus in the presence of F36 (50 μM) at 4°C for 1 h, washed to remove the unadsorbed virus and F36, and incubated at 37°C for 10 h, the release of influenza virus in the medium little reduced but it was not significant (Table 1). The result indicates that F36 did not inhibit the adsorption of influenza virus to MDCK cells. When F36 was added at 0 h or 2 h after incubation, the release of virus in the medium and MDCK cell-associated sialidase activity were reduced in comparison with control (Table 1). When F36 was added at 0 h and removed at 2 h or 3 h after the incubation, infectious virus was reduced in the medium. These results indicate that F36 inhibits the early stage(s) of influenza virus replication cycle in MDCK cells. Whereas, when the flavone


A
B
C
D

was added at 3 h or 4 h, the infectious virus in the medium was reduced but the cell-associated virus sialidase activity was increased, compared to that of control cells (Table 1). When F36 was added at 3 h and removed at 6 h after incubation, infectious virus titer in the medium also reduced. These results suggest that F36 inhibits the release of the virus from the cells at 3 h to 6 h after incubation. When F36 was added at 6 h, both the release of virus in the medium and cell-associated virus sialidase activity were not affected (Table 1).

3.3. Scanning electron microscopy (SEM) of infected MDCK cells following treatment with F36

MDCK cells which adsorbed with mouse-adapted influenza virus A/PR/8/34 were incubated in the presence or absence of F36 (50 μM) from 3 h to 9 h, and examined by SEM. As shown in Fig. 2B, numerous budding virus particles (arrows) were observed on the surface and microvilli of the MDCK cells in the absence of F36. Whereas little budding virus particles were shown on the surface of cells in the presence of F36 (Fig. 2D). These findings suggest that F36 inhibits the budding of progeny viruses from the surface of MDCK cells. SEM also revealed that F36 (50 μM) suppressed the appearance of microvilli on the surface of MDCK cells (Fig. 2C). Same concentration of F36 showed little effect on the viability of the cells by MTT assay as described above. These results indicated that F36 suppressed the appearance of microvilli without affecting viability of MDCK cells.

3.4. Effect of F36 on the distribution pattern of viral antigen in MDCK cells

The distribution pattern of viral antigen was examined by the immunofluorescence method and TIEM. As shown in Fig. 3A, MDCK cells revealed a finely dotted distribution of viral antigen in the absence of F36 by the cytoplasmic immunofluorescence method. In the presence of F36, however, the viral antigen showed different distribution pattern because the fluorescence was enriched around the cell surface and the aggregated spots of fluorescence were observed (Fig. 3B). When the surface of MDCK cells were stained with antiviral antibody, the numerous budding of the virus was observed on the surface of MDCK cells by TIEM and viral antigen was predominantly located on the virus particle in the absence of F36 (Fig. 4A, arrows). But, in the presence of F36 a small number of budding of the virus was observed on the surface of MDCK cells and the viral antigen was distributed on the cell surface (Fig. 4B, line). These findings suggest that F36 cause the accumulation of viral antigen on the MDCK cell surface by inhibition of budding of progeny viruses.

Fig. 2. Scanning electron micrographs of MDCK cells infected with influenza virus in the presence or absence of F36. MDCK cells grown on the cover slips were adsorbed with mouse-adapted influenza virus A/PR/8/34 at a MOI of 10 PFU/cell (B and D) or mock-infected (A and C) and incubated at 37°C. At 3 h, F36 (50 μM) (C and D) or its solvent (A and B) was added to the cells and incubated for up to 9 h. Thereafter, the cells were processed for SEM as described in Section 2. Arrows indicate virus particles on the cell surface and microvilli. Magnification of each photograph is $\times$30000.

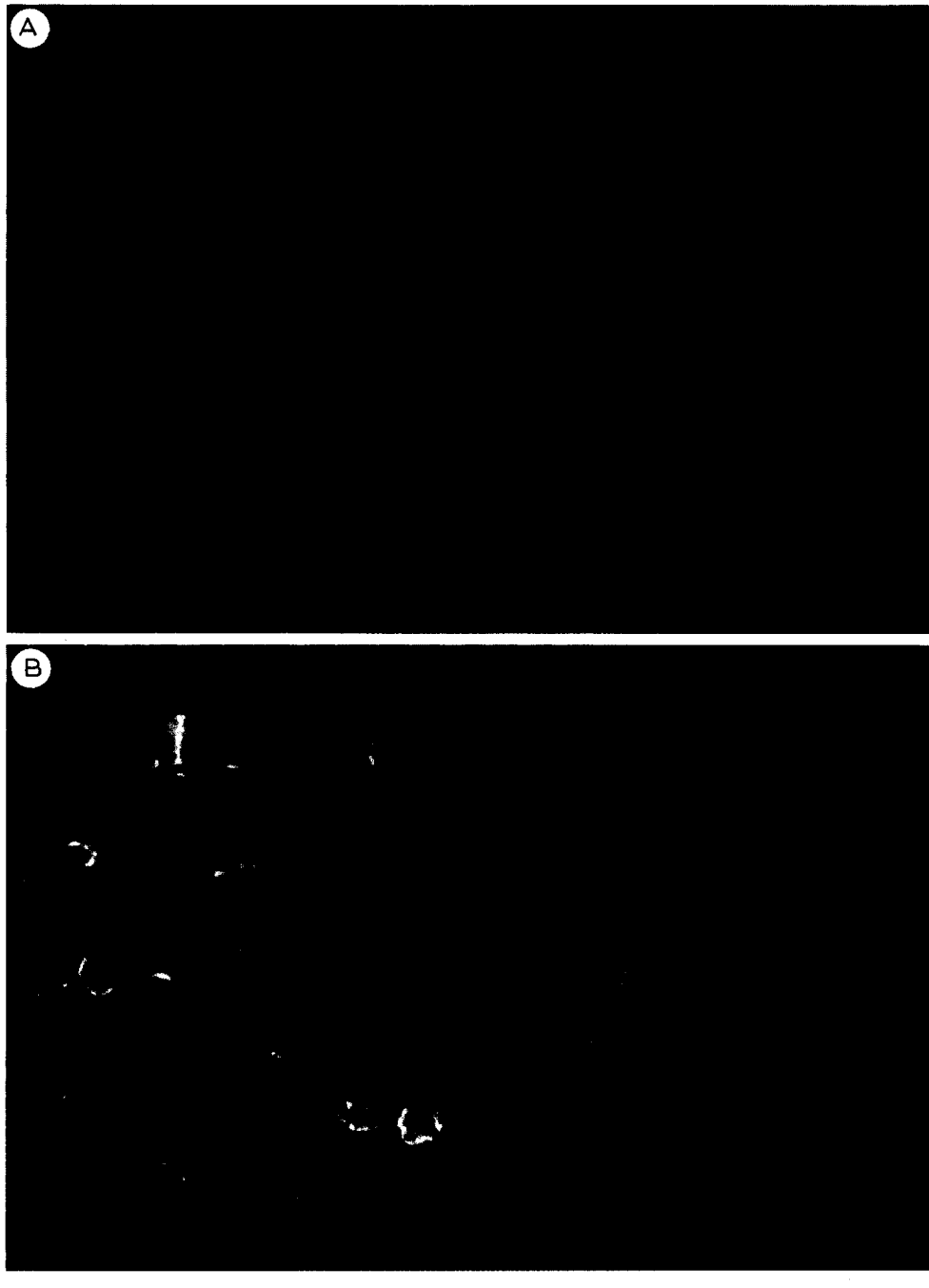


Fig. 3. Immunofluorescence micrographs of MDCK cells infected with influenza virus in the presence or absence of F36. MDCK cells grown on the cover slips were infected and cultured in the absence (A) or presence of F36 (50 μM) (B) as described in the legend to Fig. 2. The cells were fixed, and stained with mouse anti-whole virus antibody and FITC-labeled anti-mouse IgG antiserum. Magnification of each photograph is $\times 400$.

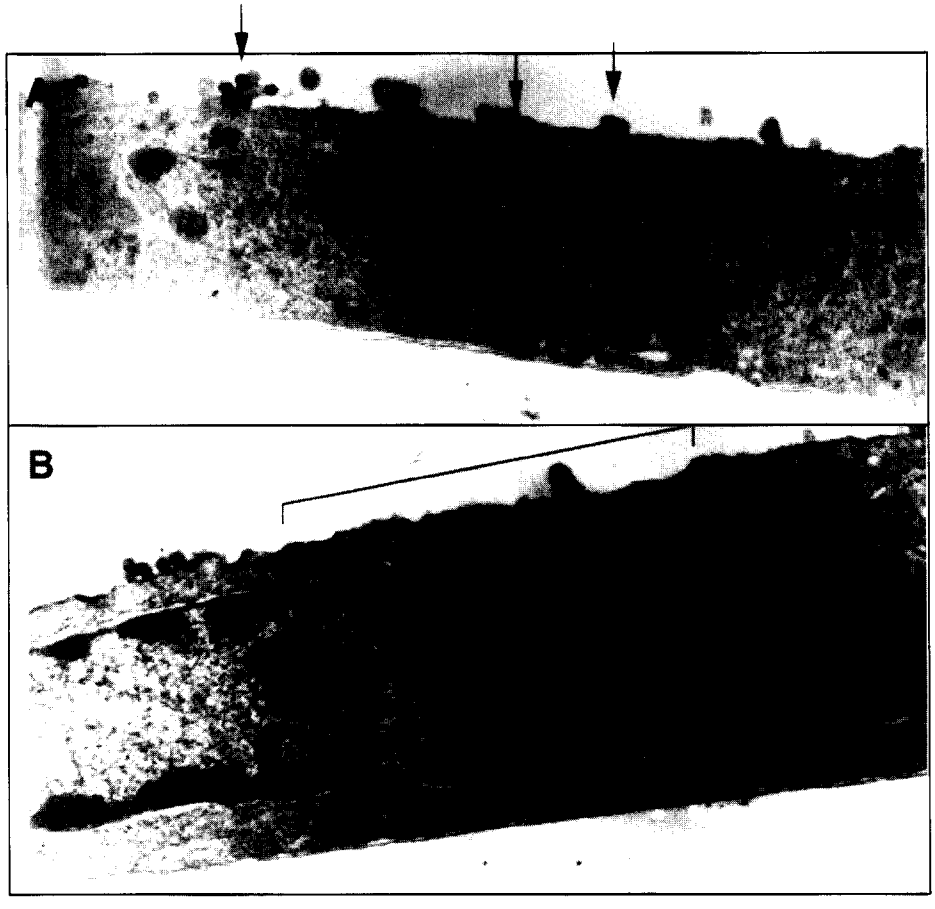


Fig. 4. Transmission immunoelectron micrographs of MDCK cells infected with influenza virus in the presence or absence of F36. MDCK cells grown on the culture dishes were infected and cultured in the absence (A) or presence of F36 (50 μM) (B) as described in Section 2. The cells were stained with mouse anti-whole virus antibody and peroxidase-labeled anti-mouse IgG antiserum. Arrows (A) indicate virus particles and line (B) indicates virus antigen on the cell surface. Magnification of each photograph is $\times 30000$.

3.5. Effect of F36 on the fusion of influenza virus with liposome

When R 18-labeled mouse-adapted influenza virus A/PR/8/34 preparation (2.6×10^7 PFU) was incubated with the liposomes containing gangliosides in the absence of F36, and pH was adjusted to 5, the time-dependent increase of fluorescence (expressed in % FDQ) was observed by the fusion. But the time-dependent increase of %FDQ by the fusion of the virus with liposome was reduced in the presence of 120 μM (37 μg/ml) of F36 (Fig. 5A). Dextran sulfate (30 μg/ml) also reduced the fusion of the virus with liposome, but the degree of inhibition was smaller than that of F36 (Fig. 5B).

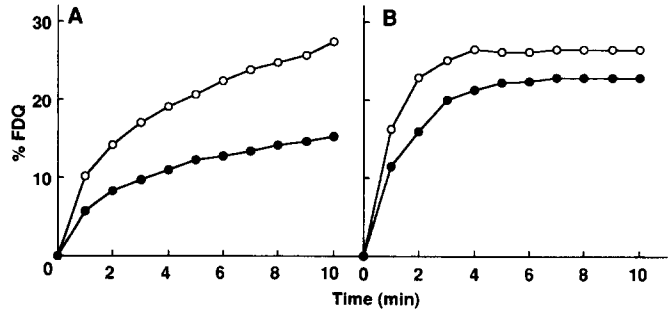


Fig. 5. Effect of F36 on the fusion of influenza virus with liposomes containing gangliosides. The increase of fluorescence by the fusion of R 18-labeled mouse-adapted influenza virus A/PR/8/34 with liposomes containing gangliosides was expressed as % fluorescence dequenching (FDQ) in the absence (○) or presence of F36 (120 μM) (●), and (B) in the absence (○) or presence of dextran sulfate (30 μg/ml) (●) ($N = 3$). 100% FDQ was obtained by addition of Triton X-100.

These results indicate that F36 inhibits the fusion of the virus with liposomes containing ganglioside.

3.6. Effect of F36 on RNA polymerase

The effect of F36 on influenza virus RNA-dependent RNA polymerase activity was examined in vitro. F36 showed little inhibition on the elongation activity of RNA polymerase of mouse-adapted influenza viruses A/PR/8/34 at even 300 μM (data not shown).

4. Discussion

In the present study, 5,7,4′-trihydroxy-8-methoxyflavone (F36) from the roots of *S. baicalensis* showed anti-influenza virus activity on single-cycle replication of mouse-adapted influenza viruses A/PR/8/34 in MDCK cells.

When F36 was added at 0 h or 2 h after incubation (the early stage of virus replication cycle), both the release of virus in the medium and cell-associated virus were reduced. F36 showed no effect on the viral adsorption to the cells and little inhibitory activity against viral RNA-dependent RNA polymerase. However, F36 significantly inhibited the fusion of influenza virus with liposomes containing ganglioside at acidic pH. These results suggest that F36 shows anti-influenza virus activity on the early stage of virus replication by inhibiting the fusion of the virus with endosome/lysosome membrane.

Wharton et al. (1989) reported that the hemagglutinin initiates the fusion process by inserting its hydrophobic amino acid sequences into the lipid bilayer of the host-cell endosome/lysosome membrane (1989). Therefore F36 may interact with the hydrophobic region of hemagglutinin and inhibits the fusion process, because F36 has hydrophobic structure. It has a possibility that F36 inhibits the fusion of influenza virus by

interaction to viral sialidase, because Huang et al. (1980); Huang et al. (1985) observed that sialidase is also required for the fusion of influenza virus.

When F36 was added to the MDCK cells infected with mouse-adapted influenza virus A/PR/8 at 3 h or 4 h after incubation (the budding stage of virus replication cycle), release of infectious virus in the medium was reduced but cell-associated virus was increased. A small number of budding of virus particles was observed on the cell surface and microvilli by SEM and TIEM, when F36 was added at the budding stage. Immunofluorescence microscopy and TIEM also showed that the accumulation of the viral antigen on MDCK cell surface by the action of F36. These results indicate that F36 shows anti-influenza virus activity by inhibiting the budding of progeny virus, when F36 was added at the budding stage after the fusion stage of influenza virus replication cycle. If F36 inhibits the replication of virus before its budding stage, viral antigen can not be detected on the cell surface. This mechanism may affect to the viruses which are escaped from inhibition of fusion and/or multi-cycle replication of the virus. Previously, we reported that F36 has a significant inhibitory activity against influenza virus sialidase (Nagai et al., 1990; Nagai et al., 1992). Influenza virus sialidase presumably aids in the elution of newly formed viruses from the infected host cells (Murti and Webster, 1986). Therefore, it brings a possibility that F36 inhibits the budding of newly formed progeny influenza virus from the infected cells by inhibiting the virus sialidase.

Synthetic neuraminic acid analogs, such as 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) (Meindl et al., 1974), 4-guanidino-Neu5Ac2en and 4-amino-Neu5Ac2en (von Itzstein et al., 1993) have been reported to inhibit influenza virus sialidase, and to have anti-influenza virus activity in the culture cells (Palese et al., 1974; von Itzstein et al., 1993; Woods et al., 1993). When influenza virus is grown in the culture cells in the presence of FANA, aggregation of progeny virions is observed on the cell surface by TEM and the virions contain sialic acid residue (Palese and Compans, 1976). This observation was suggested that FANA inhibits influenza virus replication by preventing the removal of sialic acid from the virus envelope, which serve as receptor for other virus particles causing extensive aggregation. F36 also inhibited influenza virus sialidase (Nagai et al., 1990), but the inhibition of budding of virus was observed on the MDCK cell surface by TIEM in the presence of F36, and aggregation of virus particles was not shown. These observations suggest that FANA inhibited the removal of sialic acid residues from the virus envelope glycoproteins, but F36 inhibited the removal from host cell surface receptor glycocojugates.

In the present paper, it was clearly demonstrated that F36 shows anti-influenza virus activity by inhibiting fusion and budding processes of the virus. Relationships between inhibition of these processes and sialidase inhibitory activity by the action of F36 must be elucidated in the further studies, and these studies are now in progress.

Acknowledgements

We are grateful to Mr. T. Asahina and Mr. Y. Suzuki, JEOL Ltd. (Tokyo, Japan), for operation of scanning electron microscope. We wish to thank Ms. M. Kobayashi, Mr. T. Koide and Ms. K. Kuwabara for their technical assistance.

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