Antiviral Activity of Plant Flavonoid, 5,7,4'‐Trihydroxy‐8‐methoxyflavone, from the Roots of *Scutellaria baicalensis* against Influenza A (H3N2) and B Viruses

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We investigated effects of isoscutillearein-8-methylether (5,7,4'-trihydroxy-8-methoxyflavone, F36) from the roots of *Scutellaria baicalensis* on the single-cycle replication of mouse-adapted influenza viruses A/Guizhou/54/89 (H3N2 subtype) and B/Ibaraki/2/85 in Madin-Darby canine kidney (MDCK) cells. The agent suppressed replication of these viruses from 6 to 12 h after incubation in a dose-dependent manner by 50% at 20 μM and 90% at 40 μM, respectively. F36 (50 μM) reduced the release of B/Ibaraki virus in the medium by 90–93% when it was added to the MDCK cells at 0 to 4 h after incubation. The cell-associated virus determined by sialidase activity was also reduced by the treatment at 0 to 4 h. F36 (120 μM) inhibited the low pH-dependent membrane fusion of both the viruses with the liposome containing mixed gangliosides from bovine brain. However, the agent little affected the hemagglutination and RNA-dependent RNA polymerase activities of these viruses in vitro. These results suggest that F36 inhibits the replication of A/Guizhou and B/Ibaraki viruses at last partly by inhibiting the fusion of viral envelopes with the endosome/lysosome membrane which occurs at the early stage of the virus infection cycle. F36 (0.5 mg/kg) showed no antiviral activity against A/Guizhou and B/Ibaraki viruses in mice when administered intranasally 5 min prior to virus inoculation, whereas it significantly inhibited their proliferation in the mouse lung when administered intranasally 7 times (total 3.5 mg/kg) from 18 h before to 54 h after virus infection.

Key words flavonoid; influenza virus; antiviral activity; sialidase; action mode

Influenza viruses can be classified in three types, A, B and C, by the antigenic difference of their nucleoprotein and matrix protein.1,9 Influenza A and B viruses express two envelope glycoproteins: hemagglutinin and sialidase [neuraminidase, EC 3.2.1.18].1,11 Influenza A viruses are further subclassified into several subtypes by antigenic difference of their hemagglutinin (H) and sialidase (N), such as H1N1, H2N2 and H3N2 subtypes in human.1,3 These subtypes of influenza A virus have developed by exchanges of both the hemagglutinin and sialidase genes (antigenic shift). Minor antigenic variations (antigenic drift) have also occurred in these envelope glycoproteins of influenza A and B viruses.1,3 The antigenic variations in these two viruses are what cause outbreaks of epidemic infections of the viruses.

Amantadine and rimantadine are currently used as anti-influenza virus agents, but these are effective only for influenza A virus, not for B virus, and are not always curative even for A viruses.2,9 Therefore, development of new antiviral agents has been requested which are effective for the groups of both influenza A and B viruses. We previously reported that 5,7,4'-trihydroxy-8-methoxyflavone (F36) from the roots of *Scutellaria baicalensis* showed a specific inhibitory activity against the influenza virus sialidase but not mouse liver sialidase in vitro,3 and that the flavone also showed potent antiviral activity against an H1N1 subtype of influenza A virus, A/PR/8/34, in Madin-Darby canine kidney (MDCK) cells, in the allantoic sac of embryonated eggs and in BALB/c mice.3,4 If the active sites of influenza virus sialidase are preserved regardless of the antigenic variations, specific inhibitors of influenza virus sialidase may be useful antiviral agents against the influenza A and B virus infections. Recently, it has been reported that a new analog of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en), 4-guanidino-Neu5Ac2en, displayed specific inhibitory activity against the influenza virus sialidase and also showed antiviral activity against several subtypes of influenza A and B viruses in MDCK cells.3 This compound, however, has not yet been reported to show the antiviral activity against influenza B virus in vivo.5

The present paper describes antiviral activity of F36 against the replication of H3N2 subtype of influenza A virus and influenza B virus in vitro and in vivo. The mechanism of action of anti-influenza virus activity of F36 was also investigated.

MATERIALS AND METHODS

Materials F36 was purified from the root of *S. baicalensis*6 or synthesized according to the previously described procedures.7 Sodium p-nitrophenyl-N-acetyl-z-D-neuraminic acid (PNP-NeuAc) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Octadecyl rhodamine B-HCl (R 18), used for the labeling of influenza virus, was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). Adenyllyl(3'→5')guanosine (ApG) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and RNase inhibitor (human placenta)) was from Wako. [2,8-3H]Adenosine 5'-triphosphate (ATP) (1.0 Tbiq/mmol) was obtained from Du Pont/NEN Research Products (Wilmington, DE, U.S.A.).

Cells and Viruses MDCK cells were grown in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Mouse-adapted
influenza viruses A/Guizhou/54/89 (H3N2 subtype) and B/Ibaraki/2/85 were kindly provided by Dr. Tamura (National Institute of Health, Tokyo, Japan). The viruses were grown in the allantoic cavity of 10-d-old embryonated hen eggs at 34°C for 2 d. The allantoic fluid was harvested and clarified at 1000 × g for 20 min, and then resulting supernatant fractions were subdivided and stored at −80°C until use.

**Sialidase Assay** Influenza virus sialidase activity was assayed as previously described. In brief, the reaction mixture (0.11 ml) containing 25 nmol of PNP-NeuAc and influenza virus as the enzyme source in 25 mM citrate-phosphate buffer, optimum pH of enzyme, was incubated at 37°C for 15 min in a 96 well microtiter plate. 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 the tissue culture plate were washed with phosphate-buffered saline (PBS), pH 7.4, and 0.25 mM PNP-NeuAc in PBS (200 µl) was added to the well. The plate was incubated at 37°C for 15 min, and the p-nitrophenol in the supernatant was determined from the absorbance.

**In Vitro Anti-influenza Virus Activity** F36 was dissolved in dimethyl sulfoxide (DMSO). MDCK cells were plated in a 48 well tissue culture plate (Costar; 11.3 mm well diameter). The influenza virus was adsorbed to confluent monolayers of the cell culture at a multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell (ca. 1.1 × 10⁶ PFU) in 100 µl of PBS containing 1% bovine serum albumin (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 a 5% CO₂ atmosphere in 0.5 ml of EMEM containing 1% BSA, 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 the culture plate at an appropriate time. The infectious virus titer in the medium was determined by the PFU assay using the MDCK cells. The monolayers in the culture plate were separated from the medium, washed with PBS to remove the dead cells resulting from infection with 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 as described previously.

**Fusion Assay** F36 was dissolved 50% DMSO. Liposomes containing bovine brain mixed ganglioside as receptor for influenza virus were prepared by a detergent-dialysis method. In brief, R18-labeled purified influenza virus preparation (18 µl, 2.6 × 10⁷ 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 (F₁), 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 (Fₐ). The percentage of fluorescence-dequenching (FDQ) was calculated as follows:

\[ \% \text{FDQ} = \frac{(F - F_0)(F_a - F_0)}{F_a} \times 100 \]

**Ribonucleic Acid (RNA)-Dependent RNA Polymerase Assay** F36 was dissolved in 50% DMSO. Influenza virus RNA polymerase activity was assayed as previously described. The reaction mixture (0.11 ml) containing 30 nmol tris(hydroxymethyl)aminomethane-HCl, pH 7.7, 5 mM MgCl₂, 2.5 mM dithiothreitol, 0.1 mM NaCl, 0.1% Nonidet P-40, 0.25 mM ApG, RNase inhibitor (220 units/ml), 0.2 mM guanosine 5′-triphosphate, 0.2 mM CTP, 0.2 mM UTP, 0.2 mM [³²P]ATP (1.1 MBq/µmol); purified virus (0.15 mg protein/ml) as the enzyme and F36 solution (10 µl) was incubated at 30°C for 1 h. The RNA produced was precipitated by the addition of 0.1% BSA (0.25 ml) and a mixture of 10% TCA and 0.1% sodium pyrophosphate (1 ml) for 30 min in an ice bath. The TCA-insoluble material was spotted on the glass fiber disc (GF/C filter, Whatman) by filtration. The disc was washed with a mixture of 10% TCA and 0.1% sodium pyrophosphate, then ethanol, and dried under air. TCA-insoluble [³²P] on the disc was counted in toluene-based scintillation fluid with a liquid scintillation counter (Aloka).

**In Vivo Anti-influenza Virus Activity** Female BALB/c mice, 7-weeks-old (Japan SLC Co., Ltd., Hamamatsu-shi, Japan) were used in all experiments. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbitol (50 mg/kg), and then infected by intranasal administration of 10 µl of mouse-adapted influenza virus suspension in PBS containing 0.1% BSA (a 1/5000 suspension of the original virus pool with 10⁻⁹ 50% egg infective dose (EID₅₀) for A/Guizhou and 10⁻⁵ EID₅₀ for B/Ibaraki). F36 was dissolved in 10 mM Na₂CO₃/saline, and 10 µl of the solution was administered to the mouse intranasally. Three days later, broncho-alveolar wash was obtained by two injections of 2 ml of PBS containing 0.1% BSA and antibiotics into the trachea and lungs which were separated from the body. Serial 10-fold dilutions of the broncho-alveolar wash were prepared in maintenance medium and 100 µl of each dilution was added to the confluent monolayers of MDCK cells in the wells of a 96 well culture plate, and then incubated at 37°C for 3 d under a 5% CO₂ atmosphere. The virus titer of mouse lung was expressed as the lowest dilution of the broncho-alveolar wash which was capable of infecting the MDCK cells by cytopathic effect.

**RESULTS**

**Anti-influenza Virus Activity of F36 in MDCK Cells** Effects of F36 on single-cycle replication of mouse-adapted influenza viruses A/Guizhou and B/Ibaraki in MDCK cells were studied. When the viruses (10 MOI, ca. 1.1 × 10⁶ PFU) were adsorbed to MDCK cells at 4°C and incubated at 37°C, infectious virus was detected in the medium from 6 h after incubation and reached a plateau at 11 h (Figs.
Fig. 1. Effect of F36 on Single-Cycle Replication of Influenza Virus A/Guizhou/54/89 in MDCK Cells
MDCK cell monolayers were infected with mouse-adapted influenza virus A/Guizhou/54/89 at a MOI of 10 PFU/cell for 1 h at 4°C, washed with PBS and added with F36 solution. (A) The cells were incubated at 37°C for 12 h in the absence (○) or presence of F36 (50 μM) (●). Student’s t-test: • * p < 0.05. (B) Viable cells were determined by MTT assay (■) and the infectious virus in the supernatant was quantified by PFU assay (□) at 10 h after incubation. Values represent mean ± S.D. (n = 3).

Fig. 2. Effect of F36 on Single-Cycle Replication of Influenza Virus B/Ibaraki/2/85 in MDCK Cells
MDCK cell monolayers were inoculated with 10 MOI of mouse-adapted influenza virus B/Ibaraki/2/85, and then incubated at 37°C in the presence of F36. (A) The cells were incubated at 37°C for 12 h in the absence (○) or presence of F36 (50 μM) (●). Student’s t-test: • * p < 0.05. (B) Viable cells were determined by MTT assay (■) and the infectious virus in the supernatant was quantified by PFU assay (□) at 10 h after incubation. Values represent mean ± S.D. (n = 3).

1A, 2A). When the MDCK cells infected with A/Guizhou or B/Ibaraki virus were incubated in the presence of F36 (50 μM), in contrast, infectious virus titer was significantly reduced in the medium from 6 to 12 h (Figs. 1A, 2A). When the curative activity of F36 in the A/Guizhou and B/Ibaraki infected cultures was estimated at 10 h after incubation, 89% and 85% of the cells survived in the presence of F36 (100 μM), while 44% and 55% of MDCK cells became extinct in the untreated infected cultures, respectively (Figs. 1B, 2B). Dose-dependent reduction of infectious virus titer in the medium was observed, and a 50% virus inhibitory dose of F36 was 20 μM for A/Guizhou and a 90% virus inhibitory dose was 40 μM for B/Ibaraki (Figs. 1B, 2B). F36 showed little effect on the viability of MDCK cells at 12.5—100 μM by MTT assay (data not shown).

Time-Dependent Effects of F36 on Influenza Virus Replication in MDCK Cells
The time-dependent effects of F36 were examined against the single-cycle replication of mouse-adapted B/Ibaraki virus in MDCK cells. MDCK cells were infected with the virus at 4°C for 1 h, washed to remove the unadsorbed viruses, and then incubated at 37°C for 10 h. When F36 (50 μM) was added at 0, 1, 2 or 4 h after incubation, the release of infectious virus in the medium was reduced to 7—10% and MDCK cell-associated virus determined by sialidase activity was...

<table>
<thead>
<tr>
<th>Duration of F36 treatment</th>
<th>Infectious virus in medium (×10^4 PFU/ml)</th>
<th>Cell-associated virus (units/well as sialidase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 10 h</td>
<td>3.6 ± 1.1</td>
<td>1.02 ± 0.09</td>
</tr>
<tr>
<td>1 to 10 h</td>
<td>3.3 ± 1.1</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>2 to 10 h</td>
<td>2.6 ± 0.8</td>
<td>0.91 ± 0.12</td>
</tr>
<tr>
<td>4 to 10 h</td>
<td>3.8 ± 1.2</td>
<td>0.96 ± 0.10</td>
</tr>
<tr>
<td>6 to 10 h</td>
<td>27.3 ± 12.3</td>
<td>1.13 ± 0.06</td>
</tr>
<tr>
<td>8 to 10 h</td>
<td>26.7 ± 6.4</td>
<td>1.21 ± 0.06</td>
</tr>
<tr>
<td>Control</td>
<td>39.3 ± 5.5</td>
<td>1.35 ± 0.04</td>
</tr>
</tbody>
</table>

a) MDCK cells were infected with influenza 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. b) Values represent mean ± S.D (n = 3).

c) One unit was defined as the amount of enzyme which hydrolyzed 1 μmol of PNP-NenAc/min.

d) Virus-infected and 1% DMSO (final concentration) but not F36 treated cells.
Fig. 3. Effect of F36 on the Fusion of Influenza Virus with Liposome Containing Gangliosides

The increase of fluorescence by the fusion of R 18-labeled mouse-adapted influenza virus A/Guizhou/54/89 (A) or B/Ibaraki/2/85 (B) with liposomes containing gangliosides was expressed as % FDQ in the absence (○) or presence of F36 (120 μM) (●). 100% FDQ was obtained by addition of Triton X-100.

reduced to 67—76% in comparison with control (Table I). When the flavone was added at 6 or 8 h, however, the release of virus in the medium and the cell-associated virus were little affected (Table I). As shown in Fig. 2A, release of influenza virus B/Ibaraki in the medium begins 6 h after incubation. These results suggest that F36 inhibits the early stage(s) of replication cycle of the virus in MDCK cells.

**Effects of F36 on Hemagglutination by Influenza Virus**

The effect of F36 on hemagglutination by influenza virus was examined. F36 (0.35 mM) showed little effect on the hemagglutination titer of both the A/Guizhou (H3N2) and B/Ibaraki viruses when the flavone was mixed directly with the virus suspensions (data not shown).

**Effects of F36 on the Fusion of Influenza Virus with Liposome**

When the R 18-labeled A/Guizhou and B/Ibaraki virus preparations (2.6 x 10⁷ PFU) were incubated with liposomes containing gangliosides in the absence of F36, and pH was adjusted to 5.0, the time-dependent increase of fluorescence (expressed in %FDQ) was observed, which would be caused by the fusion activity of A/Guizhou (Fig. 3A) and B/Ibaraki viruses (Fig. 3B). The time-dependent increase of %FDQ by both viruses, however, was reduced in the presence of F36 (120 μM) (Fig. 3). These results suggest that F36 inhibits the fusion of A/Guizhou (H3N2) and B/Ibaraki viruses with the liposomes.

**Effects of F36 on RNA Polymerase**

The effect of F36 on influenza virus RNA-dependent RNA polymerase activity was examined in vitro, and little effect was shown on the activity of either virus at 300 μM (Table II).

**Anti-influenza Virus Activity of F36 in Mice**

F36 has been reported to show significant antiviral activity against mouse-adapted influenza virus A/PR/8/34 (H1N1 subtype) in mice. Accordingly, we tested the effects of F36 on A/Guizhou and B/Ibaraki virus infections in BALB/c mice. When 0.5 mg/kg of F36 was administered intranasally 5 min before the infection, the lung virus titer was not reduced for either virus (data not shown), whereas when the same administration was done 7 times (total 3.5 mg/kg) at 18 h, 5 min before and 6, 24, 30, 48 and 54 h after virus inoculation, F36 significantly reduced the titers of both viruses in the lung compared with those of control treated with solvent alone (Table III). These results indicate that F36 displays the antiviral activity against infections in vivo with the influenza A (H3N2 subtype) as well as B viruses.

**DISCUSSION**

F36 reduced the yield of infectious viruses in the medium and MDCK cell-associated viruses of both the influenza A/Guizhou and B/Ibaraki viruses when it was added earlier than 4 h after incubation. F36 showed little inhibitory effect against the viral RNA-dependent RNA polymerase activity and hemagglutination of these viruses; however, it significantly inhibited the fusions of both types of virions with the liposomes containing gangliosides at acidic pH. These results suggest the possibility that antiviral activity of F36 observed at the early stage(s) of the replicative process of A/Guizhou and B/Ibaraki viruses would be dependent at least on the inhibition of their fusions with the endosome/lysosome membrane of the host cell.

We reported that F36 has a significant inhibitory activity

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**TABLE II. Effect of F36 on Influenza Virus RNA Polymerase Activity**

<table>
<thead>
<tr>
<th>Influenza virus strain (subtype)</th>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>TCA insoluble [H]¹ᵃ (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Guizhou/54/89 (H3N2)</td>
<td>F36</td>
<td>300</td>
<td>2166 ± 215</td>
</tr>
<tr>
<td>B/Ibaraki/2/85</td>
<td>F36</td>
<td>300</td>
<td>1913 ± 62</td>
</tr>
</tbody>
</table>

¹ᵃ Values represent mean ± S.D. (n = 3).

**TABLE III. Antiviral Activity of F36 against Influenza A (H3N2) and B Viruses in Mice**

<table>
<thead>
<tr>
<th>Influenza virus strain (subtype)</th>
<th>Compound</th>
<th>Total dose (mg/kg)</th>
<th>Lung virus titer (10⁸)</th>
<th>Student's t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Guizhou/54/89 (H3N2)</td>
<td>F36</td>
<td>8.0</td>
<td>5.0 ± 0.0</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>B/Ibaraki/2/85</td>
<td>1 mm Na₂CO₃</td>
<td>3.5</td>
<td>5.0 ± 0.4</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
against influenza virus sialidase.\textsuperscript{38,44} It may inhibit the fusion activity of influenza virus by interacting with the viral sialidase, because Huang \textit{et al.} observed that sialidase was required for the fusion of influenza viruses.\textsuperscript{12} Investigation of a possible relationship between the sialidase inhibition and inhibition of the viral envelope fusion by F36 is now in progress.

F36 completely prevented proliferation of mouse-adapted influenza virus A/PR8 (H1N1 subtype) in the mouse lung when administered intranasally (0.5 mg/kg), whereas it did not inhibit proliferation of mouse-adapted influenza A/Guizhou (H3N2 subtype) and B/Ibaraki viruses in this organ under the same conditions. F36 showed significant antiviral activity against infections with these viruses when administered intranasally 7 times (total 3.5 mg/kg) from 18 h before to 54 h after virus inoculation. The antiviral activity of F36 reportedly was caused by inhibiting the early stage and budding stage of the A/PR8 virus replication.\textsuperscript{13} Accordingly, F36 may inhibit the budding stage of the mouse-adapted influenza virus A/PR8 in addition to inhibiting the early stage of the virus replication. In the present study, we showed that F36 inhibited only the early stage of the replication cycle of A/Guizhou and B/Ibaraki viruses, probably the fusion stage. These findings indicate that A/Guizhou and B/Ibaraki viruses are less sensitive to F36 than A/PR8 virus in mice, and this may be related to the mode of action of the flavonoid against these viruses.

A potent influenza virus sialidase inhibitor, F36,\textsuperscript{31} also showed antiviral activity against infection with the H1N1\textsuperscript{4} and H3N2 subtypes of influenza A virus and influenza B virus in MDCK cells and in mice. von Itzstein \textit{et al.} and Woods \textit{et al.} also reported that 4-guanidino-2,4-deoxy-2,3-dehydro-N-acetyl neuraminic acid (4-guanidino-Neu5-Ac2en) has specific inhibitory activity against the influenza virus sialidase and has potent antiviral activity against influenza A virus infections in MDCK cell cultures and \textit{in vivo}, as well as influenza B virus infections in MDCK cell cultures.\textsuperscript{5} These results indicate that influenza virus sialidase is a good target of anti-influenza virus drugs.

**Acknowledgments** We are grateful to Dr. S.-I. Tamura, National Institute of Health (Tokyo, Japan), for providing mouse-adapted influenza viruses A/Guizhou/54/89 and B/Ibaraki/2/85. We also wish to thank Ms. S. Harakawa and Mr. H. Sakaniwa for their technical assistance.

**REFERENCES**