Antiviral Research, 19 (1992) 207–217

AVR 00582

In vivo anti-influenza virus activity of plant flavonoids possessing inhibitory activity for influenza virus sialidase

Takayuki Nagai[a], Yukinori Miyaichi[c], Tsuyoshi Tomimori[c], Yujiro Suzuki[b] and Haruki Yamada[a]

[a]*Oriental Medicine Research Center and* [b]*Department of Virology, The Kitasato Institute, Minato-ku, Tokyo 108, Japan and* [c]*School of Pharmacy, Hokuriku University, Kanazawa, Ishikawa 920-11, Japan*

(Received 24 May 1991; accepted 22 May 1992)

Summary

Isoscutellarein (5,7,8,4'-tetrahydroxyflavone) from the leaf of *Scutellaria baicalensis* non-competitively inhibited (IC_{50}, 20 μM) the hydrolysis of sodium *p*-nitrophenyl-*N*-acetyl-α-D-neuraminate by influenza virus sialidase with an apparent K_i value of 41 μM. Negligible inhibitory activity was observed for mouse liver sialidase at a concentration of 79 μM. Isoscutellarein also inhibited the replication of influenza virus A/WSN/33 in Madin-Darby bovine kidney cells with 50% virus inhibitory dose at 16 nmol/well and influenza virus A/PR/8/34 in the allantoic sac of embryonated egg with little toxic effects. The flavone showed significant anti-influenza virus activity in vitro similar to isoscutellarein-8-methylether (F36) (Nagai, T., Miyaichi, Y., Tomimori, T., Suzuki, Y. and Yamada H., 1990, Chem. Pharm. Bull. 38, 1329–1332), and more potent virucidal activity in ovo than F36. However, F36 completely prevented proliferation of mouse-adapted influenza virus A/PR/8/34 in mouse lung by the intranasal (0.5 mg/kg) and intraperitoneal (4 mg/kg) administrations, and it was more potent than the known anti-influenza virus substance, amantadine. Intranasal administration of F36 (0.5 mg/kg) also protected mice against a lethal influenza virus A/PR/8/34 infection. Isoscutellarein significantly inhibited lung virus proliferation when administered intranasally or orally to mice. F36 and isoscutellarein showed negligible toxic effect against mice. These results suggested that flavones, which have potent influenza virus sialidase inhibitory activity, have anti-influenza virus activity in vivo.

Correspondence to: H. Yamada, Oriental Medicine Research Center of the Kitasato Institute, 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan. Fax: (81) 3-3440-3992.

Influenza virus; Sialidase; Neuraminidase; Flavonoid

Introduction

Influenza A and B viruses express two envelope glycoproteins: hemagglutinin and sialidase [neuraminidase, enzyme classification EC 3.2.1.18]. The hemagglutinin is known to mediate the attachment of the virus to the host cells via sialic acid residue in glycoconjugate receptors, and the subsequent fusion of viral and host cell membranes (Wiley and Skehel, 1987). While the sialidase catalyses cleavage of terminal sialic acid residue from the sialoglycoconjugate receptors (Gottschalk et al., 1972), and presumably aids in the elution of newly formed viruses from the infected host cells and in the destruction of sialic acid containing mucus glycoproteins that can act as receptor analogs located on the host cell surface (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) from the root of *Scutellaria baicalensis* was shown to have a potent inhibitory activity against influenza virus sialidase, and that this flavone also showed anti-influenza virus activity in Madin-Darby canine kidney cells and in the allantoic sac of embryonated egg (Nagai et al., 1990). The present paper describes the inhibitory activity against influenza virus sialidase and anti-influenza virus activity in culture cells and embryonated egg by 8-hydroxyflavone of F36, isoscutellarein, and in vivo anti-influenza virus activity using BALB/c mice by F36 and isoscutellarein.

Materials and Methods

Materials

F36 (Tomimori et al., 1982) and isoscutellarein (Miyaichi et al., 1988) were isolated 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). Mouse liver sialidase was prepared as previously described (Nagai and Yamada, 1988). Other PNP-glycopyranosides, jack bean α-mannosidase, Brewer's yeast α-glucosidase, almond β-glucosidase, and amantadine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Jack bean β-galactosidase was from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan).

Cells, viruses and vaccine

Madin-Darby bovine kidney (MDBK) cells were grown in Eagle's minimum essential medium (EMEM) containing 10% non-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. For assays, cells were plated in 48 wells plastic tissue culture plates (Costar; 11.3 mm well diameter). Influenza HA vaccine and influenza viruses A/PR/8/34 (H1N1), A/WSN/33 (H1N1) and mouse-adapted A/PR/8/34 (H1N1) were obtained from the Kitasato Institute (Tokyo, Japan). Influenza viruses A/PR/8/34 and mouse-adapted A/PR/8/34 were grown in allantoic sacs of 10-day-old embryonated eggs for 48 h at 34°C, and A/WSN/33 was propagated in monolayers of MDBK cells for 72 h at 37°C in EMEM containing 2% non-inactivated FBS and antibiotics (maintenance medium). The allantoic fluid and medium each was harvested and clarified at 1000 × *g* for 20 min, and then the resulting supernatants were stored in small portions at −80°C.

Sialidase assay

Flavones were dissolved in 50% dimethyl sulfoxide (DMSO). Influenza virus sialidase activity was assayed as previously described (Nagai et al., 1990). In brief, the reaction mixture containing 25 nmol of PNP-NeuAc, flavone solution (10 μl), and influenza HA vaccine as the enzyme in 25 mM citrate-phosphate buffer, pH 5.0, 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 Micro Plate Reader Model MPR-A4 (Tosoh). Sialidase activity of mouse liver was assayed as previously described (Nagai et al., 1989).

Other glycosidase assay

Jack bean α-mannosidase, jack bean β-galactosidase, almond β-glucosidase and Brewer's yeast α-glucosidase activities were assayed with appropriate PNP-glycopyranosides as previously described (Nagai et al., 1989).

In vitro anti-influenza virus experiments

Confluent monolayers of MDBK cell cultures were infected with influenza virus A/WSN/33 at a multiplicity of infection (MOI) of 0.002 plaque forming units (PFU)/cell in 0.5 ml of maintenance medium, then the paper disc (thick type, 8 mm diameter) which absorbed flavone methanol solution and dried to remove solvent in air was put into the well of a culture plate. The paper disc which absorbed methanol was dried and used for control. The plate was cultured at 34°C for 72 h under 5% CO_2 atmosphere in the dark with occasional gentle shaking. Then the monolayers in the culture plate were separated from the medium, washed with phosphate-buffered saline (PBS), pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ to remove the dead cells resulting from infection of the influenza 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). After discarding the supernatants, 100 μl of MTT (1 mg/ml) in maintenance medium was added and the monolayers were incubated for 2 h at 37°C. The resulting formazan precipitate was dissolved with

isopropanol containing 0.04 N HCl, and the absorbance of the solution was determined spectrophotometrically at 570 nm with Micro Plate Reader MPR-A4. Anti-influenza virus activity was also estimated by the hemagglutination assay of the medium using chicken erythrocytes as previously described (Nagai et al., 1990).

In ovo anti-influenza virus experiments

The mixture of 0.2 ml of influenza virus A/PR/8/34 suspension (7×10^3 PFU/ml) and 0.1 ml of flavone 50% DMSO solution was injected into the allantoic sac of 10- to 11-day-old embryonated eggs, and then the eggs were incubated at 34°C for 48 h. Allantoic fluid was harvested, and the amount of virus in the fluid was determined by hemagglutination assay and by sialidase activity with PNP-NeuAc.

In vivo anti-influenza virus experiments

Female BALB/c mice, 6-week-old (Japan SLC Co., Ltd., Hamamatsu, Japan) were used in all experiments. Mice were anesthetized by an intraperitoneal injection of amobarbital sodium (0.25 ml of a saline solution of 11 mg/ml), and then infected by intranasal administration of 10 μl mouse-adapted influenza virus A/PR/8/34 suspension in 0.1% bovine serum albumin (BSA) in PBS (a 1/5000 suspension of the original virus pool with $10^{8.5}$ EID_{50}). For intranasal treatment, flavones were dissolved in 10 mM Na_2CO_3/saline, and 10 μl of the solution was administered to the mouse at 5 min before infection of virus. For intraperitoneal and oral administrations, flavone was suspended in 0.1% (w/v) sodium carboxymethylcellulose (CMC · Na) solution, and then the suspension was administered to the mouse at each 0.2 ml/mouse 1 h before and 3 times during 48 h after intranasal infection of the virus according to the time schedule of amantadine (Grunert et al., 1965). Three days later, the lungs were excised and homogenized in PBS to give a 10% (w/v) suspension with 6 strokes of a glass/Teflon homogenizer, and then the homogenate was centrifuged at 3500 rpm for 20 min. Serial 10-fold dilutions of the supernatant of individual lung homogenate were prepared and 0.1 ml of each dilution was injected into the allantoic sac of 10-day-old embryonated egg, and then the eggs were incubated at 34°C for 48 h. The amount of influenza virus in allantoic fluid was determined by the sialidase activity and hemagglutination assay, and the virus titer of mouse lung was expressed as the lowest dilution of the supernatant which is capable of infection in the egg. Survival of mice in groups (each 20 mice), which were infected with the mouse-adapted influenza virus A/PR/8/34, was estimated over a 15 day period after intranasal administration of F36. F36 (0.5 mg/kg) was administered to the mouse 5 min prior to virus exposure as described above.

Results

Influenza virus sialidase inhibitory activity of isoscutellarein

Isoscutellarein (5,7,8,4'-tetrahydroxyflavone), from the leaf of *S. baicalensis*, was tested for inhibitory effect on influenza virus sialidase activity using the PNP-NeuAc as substrate. Isoscutellarein inhibited the enzyme in a dose-dependent manner (Fig. 1A), and 50% of the influenza virus sialidase activity was inhibited in the presence of 20 μM of this flavone (IC_{50}). The activity was about 2.5 times more potent than isoscutellarein-8-methylether (F36) (IC_{50}, 55 μM). The initial velocity (v) of influenza virus sialidase activity was measured at increasing PNP-NeuAc concentration in the presence or absence of 40 μM of isoscutellarein, then the initial concentrations of PNP-NeuAc ($[S]_0$) were plotted against $[S]_0/v$. Isoscutellarein decreased the maximal velocity (V_{max} of influenza virus sialidase, but did not significantly alter the K_m value of the enzyme for PNP-NeuAc (Fig. 1B). This result indicated that isoscutellarein

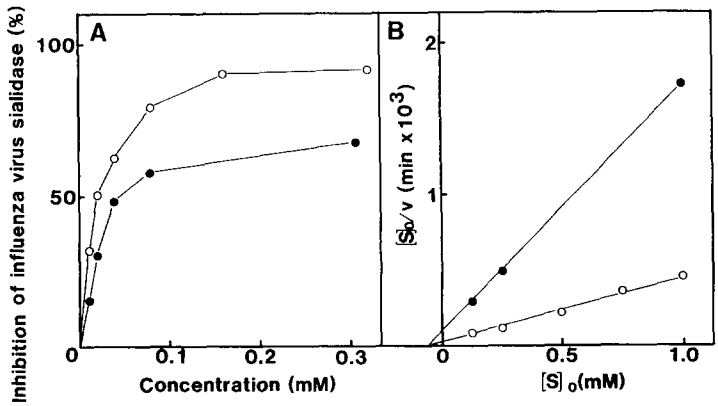

Fig. 1. Inhibitory activity of isoscutellarein against influenza virus sialidase. (A) Sialidase inhibitory activities of isoscutellarein (○) was compared with that of F36 (●) under the condition described in Materials and Methods. (B) Initial velocities of influenza virus sialidase activity were determined in the absence (○) or presence of isoscutellarein at 40 μM (●) in increasing PNP-NeuAc concentration, then $[S]_0/v$ were plotted against $[S]_0$.

TABLE 1

Inhibitory activities of isoscutellarein against influenza virus sialidase and other glycosidases

Glycosidase	Inhibition (%)[a]
Sialidase (influenza virus)	93.8
Sialidase (mouse liver)	0
β-Galactosidase (jack bean)	17.4
α-Mannosidase (jack bean)	16.0
α-Glucosidase (yeast)	42.9
β-Glucosidase (almond)	35.3

[a]Concentration of isoscutellarein; 79 μM.

inhibited influenza virus sialidase non-competitively the same as F36 (Nagai et al., 1990). The inhibition constant (K_i value) of isoscutellarein calculated using the values of V_{max} obtained at 0 and 40 μM in this assay was 41 μM. Isoscutellarein (79 μM) showed negligible inhibition of mouse liver sialidase and little inhibition of jack bean β-galactosidase and jack bean α-mannosidase, but the same concentrations of isoscutellarein inhibited other glycosidases such as yeast α-glucosidase and almond β-glucosidase, less than influenza virus sialidase (Table 1).

Anti-influenza virus activity of isoscutellarein in culture cells and egg

The effect of isoscutellarein on replication of influenza virus A/WSN/33 in MDBK cells was studied by inoculating monolayers on wells of plastic culture plate. Although 88% of MDBK cells became extinct by the infection in the absence of this flavone, in the presence of isoscutellarein (100 nmol/well) 87% of cells survived compared with the mock-infected solvent control, and dose-dependent reduction of hemagglutination titer was observed in the presence of

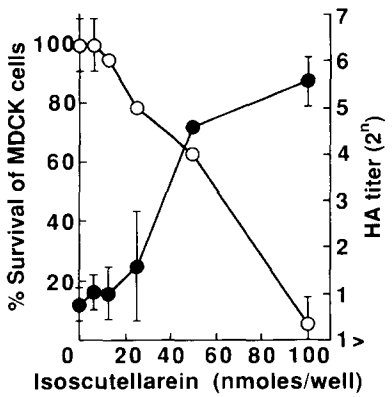

Fig. 2. Anti-influenza virus activity of isoscutellarein in MDBK cells. MDBK cell monolayers were infected with influenza virus A/WSN/33 at a MOI of 0.002 PFU/cell and added paper disc which adsorbed flavone solution was put into the well. The cells were incubated 72 h in the presence of the flavone. Viable cells were determined by MTT assay (●) and supernatant virus was quantified by hemagglutination titer (○). Values represent mean ± S.D. ($n = 3$).

TABLE 2

Anti-influenza virus activity of isoscutellarein in egg

Flavone[a]	Hemagglutination titer[b] (units/ml)	Sialidase activity[b] (munits/ml)[c]
DMSO	$2^{7.3 \pm 0.0}$	71.0 ± 15.7
Isoscutellarein	$2^{<1}$	0.0 ± 0.0
F36	$2^{5.6 \pm 1.5}$	24.6 ± 12.5

[a]Dose, 100 μg (0.35 μmol as isoscutellarein or 0.33 μmol as F36)/egg.
[b]Values represent mean ± S.D. ($n = 3$).
[c]One unit was defined as the amount of enzyme which hydrolyzed 1 μmol of PNP-NeuAc/min.

TABLE 3

Anti-influenza virus activity of flavones in mice

Compound	Dose (mg/kg)	Lung virus titer[a] (10^n)	Incidence of infection[b] (%)
Experiment 1			
Intranasal administration			
10 mM Na_2CO_3	–	$10^{6.9\pm0.8}$	8/8 (100)
F36	0.5	$10^{<2****}$	0/5 (0)
	0.15	$10^{5.7\pm0.6**}$	3/3 (100)
	0.05	$10^{6.0\pm1.0}$	3/3 (100)
Isoscutellarein	0.5	$10^{5.0\pm0.9***}$	6/6 (100)
	0.15	$10^{5.0\pm1.7**}$	3/3 (100)
	0.05	$10^{6.3\pm0.6}$	3/3 (100)
Amantadine	0.5	$10^{4.5\pm2.3**}$	4/6 (67)
	0.15	$10^{6.3\pm0.6}$	3/3 (100)
	0.05	$10^{6.7\pm0.6}$	3/3 (100)
Experiment 2			
Intraperitoneal administration			
0.1% CMC·Na	–	$10^{3.3\pm1.2}$	3/3 (100)
F36	400	$10^{<2*}$	1/3 (33)
	40	$10^{<2*}$	0/3 (0)
	4	$10^{<2*}$	0/3 (0)
Isoscutellarein	400	$10^{3.7\pm0.6}$	3/3 (100)
	40	$10^{3.7\pm0.6}$	3/3 (100)
	4	$10^{2.0\pm1.7}$	1/3 (33)
Amantadine	400	$10^{3.0\pm1.0}$	3/3 (100)
	40	$10^{3.7\pm0.6}$	3/3 (100)
	4	$10^{2.7\pm1.5}$	2/3 (67)
Experiment 3			
Oral administration			
0.1% CMC·Na	–	$10^{5.7\pm0.6}$	3/3 (100)
F36	400	$10^{5.0\pm0.0}$	3/3 (100)
	40	$10^{4.7\pm1.2}$	3/3 (100)
	4	$10^{4.7\pm0.6}$	3/3 (100)
Isoscutellarein	400	$10^{4.0\pm0.0**}$	3/3 (100)
	40	$10^{4.3\pm0.6**}$	3/3 (100)
	4	$10^{5.0\pm1.0}$	3/3 (100)
Amantadine	400	$10^{4.0\pm0.0**}$	3/3 (100)
	40	$10^{4.0\pm0.0**}$	3/3 (100)
	4	$10^{4.3\pm0.6**}$	3/3 (100)

[a]Values represent mean ± S.D. of lung homogenate endpoint titers.

[b]The presence of virus in the lung was determined as described in Materials and Methods. Values represent the number of infected mice, in which the virus was present in the lung homogenate (1:100), per tested mice. Student's *t*-test: $^{*}P<0.1$, $^{**}P<0.05$, $^{***}P<0.01$, $^{****}P<0.001$.

isoscutellarein with 50% virus inhibitory dose at 16 nmol/well (Fig. 2). These results indicate that isoscutellarein inhibited the replication of influenza virus A/WSN/33 in MDBK cells. The inhibition of replication of this virus was observed when the paper disc which absorbed flavone was added on the well, but not when added as solution (unpublished data). Isoscutellarein showed little effect on the viability of MDBK cells at 100 nmol/well by MTT assay (data not shown). Isoscutellarein also inhibited replication of influenza virus A/

PR/8/34 in the allantoic sac of embryonated egg at 100 μg (0.35 μmol)/egg completely, because negligible hemagglutination titer and sialidase activity, which are caused by residual virus, were observed in the allantoic fluid at 48 h after application of virus (Table 2). This activity was more potent than that of F36 (Table 2). Isoscutellarein and F36 (each 0.35 mM) had a little effect on hemagglutination titer of influenza virus A/PR/8/34 when each flavone was mixed directly with the virus. The injection of these flavones and their solvents to 10-day-old embryonated eggs had no effect on the percentage of hatch of the eggs compared with untreated eggs.

Anti-influenza virus activity of F36 and isoscutellarein in mice

Effects of F36 and isoscutellarein on mouse-adapted influenza virus A/PR/8/34 infections in BALB/c mice were tested. The lung virus titers determined by sialidase activity were completely parallel with the titers determined by hemagglutination assay in all experiments. Both flavones were not contained in the supernatant of lung homogenate from mouse administered flavone because the supernatant showed no influenza virus sialidase inhibitory activity. When 0.5 mg/kg of F36 was administered intranasally, the lung virus titer was reduced to $10^{<2}$ and the infection by influenza virus as determined by presence of detectable virus in the lung was completely prevented at 3 days after virus inoculation (Table 3). Isoscutellarein (0.5 and 0.15 mg/kg) and amantadine (0.5 mg/kg) also significantly inhibited virus replication in lung of mouse, but F36 showed more potent anti-influenza virus activity than isoscutellarein and the known anti-influenza viral agent, amantadine. Intranasal treatment with 0.5 mg/kg of F36 significantly improved the survival of mice inoculated with mouse-adapted A/PR/8/34 virus at 15 days (Fig. 3). F36, isoscutellarein, amantadine (400, 40 and 4 mg/kg as total dose) or 0.1% CMC·Na saline solution was administered intraperitoneally at 1 h before and 7, 24 and 48 h after challenge of mouse-adapted influenza virus A/PR/8/34. F36 reduced the

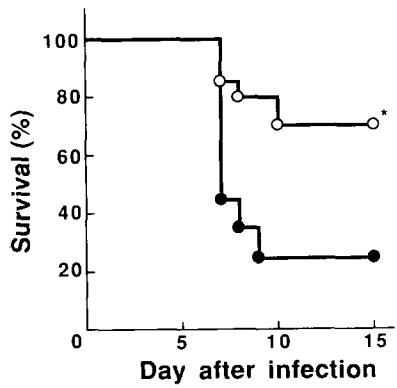

Fig. 3. Effect of F36 on the survival of mice infected with mouse-adapted influenza virus A/PR/8/34. BALB/c mice were treated i.n. with F36 (0.5 mg/kg) or Na_2CO_3/saline 5 min prior to virus exposure. Placebo: (●); F36: (○) ($n=20$). Wilcoxon test: $^*P<0.01$.

lung virus titers to $10^{<2}$ at all doses tested, and the infection by influenza virus as determined by presence of detectable virus in the lung was completely prevented at the doses of 4 and 40 mg/kg (Table 3), whereas intraperitoneal administration of isoscutellarein and amantadine did not reduce lung virus titers significantly. It appeared that anti-influenza virus activity of F36 was more potent than that of amantadine by intraperitoneal administration. When F36, isoscutellarein, amantadine (400, 40 and 4 mg/kg as total dose) or 0.1% CMC · Na solution was administered orally to mice at 1 h before and 5, 24 and 47 h after infection of the virus, F36 showed no inhibitory effect on proliferation of influenza virus in mouse lung (Table 3), but isoscutellarein significantly reduced lung virus titers at 400 and 40 mg/kg compared with 0.1% CMC · Na. This activity was almost similar with that of amantadine. In order to discover the toxicity of F36, isoscutellarein and their solvents (10 mM Na_2CO_3 and 0.1% CMC · Na saline solution) in mouse, these samples were administered to the mouse as described above except infection of influenza virus. These mice were observed for 51 days after administration of samples, and all mice survived and no significant decrease of the body weight was observed. When these mice were dissected at 51 days after administration of samples, their brain, heart, lung, liver, kidney, stomach, intestine and spleen did not show any change compared with untreated mice.

Discussion

Isoscutellarein, 8-hydroxyflavone of F36, showed more potent influenza virus sialidase inhibitory activity than F36, and also showed significant anti-influenza virus activity in MDBK cells and allantoic sac of embryonated egg. F36 inhibited proliferation of mouse-adapted influenza virus A/PR/8/34 in mouse lung by intranasal and intraperitoneal administrations almost completely. Intranasal administration of F36 improved the survival of mice infected by the virus, while isoscutellarein showed significant anti-influenza virus activity by intranasal and oral administrations. Several natural sialidase inhibitors such as 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Veh and Schauer, 1978), siastatin B (Umezawa et al., 1974), panosialin (Aoyagi et al., 1971) and neuraminin (Lin et al., 1977) and synthetic sialidase competitive inhibitors, such as analog of neuraminic acid, 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) (Meindl et al., 1974) and thioglycoside-analogs of gangliosides (Suzuki et al., 1990) have been reported. Although FANA has been reported to have anti-influenza virus activity in the culture cells (Palese et al., 1974), no sialidase inhibitor have been reported to have potent anti-influenza virus activity in vivo. Most sialidase inhibitors inhibit not only influenza virus sialidase but also mammalian sialidases. It is known that sialidase catalyzes the removal of sialic acid residues from sialoglycoconjugates, and this removal is associated with several important biological reactions (Schauer, 1982; Reutter et al., 1982), such as clearance of serum sialoglyco-

proteins, antigenic expression, and recognition by receptors. Therefore, administration of these sialidase inhibitors to mammal may be disadvantageous for these reactions. We have screened inhibitors against influenza virus sialidase from several flavonoids, and F36 which has specific inhibitory activity for influenza virus sialidase but little activity against mouse sialidase was obtained (Nagai et al., 1990). In the present study, we also find that isoscutellarein showed more potent inhibitory activity for influenza virus sialidase than F36, and showed negligible inhibitory activity for mouse liver sialidase. These flavones showed significant anti-influenza virus activity in vitro (Nagai et al., 1990), in ovo and in vivo with little or negligible toxicity in MDBK cells, embryonated eggs and mice. These results suggest that F36 and isoscutellarein should prove to be useful and specific anti-influenza virus agents by inhibiting the virus sialidase. Isoscutellarein showed more potent influenza virus sialidase inhibitory activity than F36. While F36 showed more potent anti-influenza virus activity in vivo on intranasal and intraperitoneal administrations. It may be related to the solubilities, metabolic rates and/or permeability into the infected cells of these flavones. When isoscutellarein was injected intraperitoneally, dose-dependent reduction of lung virus titer was not observed. It also may be related to the different absorption rate due to how this flavone is soluble in the body. In the present results, it is not known whether anti-influenza virus activity of F36 and isoscutellarein is a prophylactic and/or therapeutic activity. Influenza vaccine is useful for prophylaxis of influenza virus infection, but antigenicity of influenza virus is often alterable by antigenic shift and antigenic drift (Webster and Laver, 1975). If the enzymic active sites (interactive sites of inhibitor) of influenza virus sialidase is fixed against antigenic shift and antigenic drift, influenza virus sialidase specific inhibitor may become common anti-influenza viral agents for several subtypes of the virus. Investigations of sialidase inhibitory activity and anti-influenza virus activity for other subtypes of influenza A virus and influenza B virus, as well as the mechanism of action of these flavones, are now in progress.

Acknowledgements

We are grateful to Dr. T. Nagamine, Department of Technology of the Kitasato Institute, for his valuable advice and help in the infection of influenza virus to the mouse. We also wish to thank Ms. S. Harakawa for her technical assistance.

References

Aoyagi, T., Yagisawa, M., Kumagai, M., Hamada, M., Okami, Y., Takeuchi, T. and Umezawa, H. (1971) An enzyme inhibitor, panosialin, produced by Streptomyces. I. Biological activity, isolation and characterization of panosialin. J. Antibiotics 24, 860–869.

Gottschalk, A., Belyavin, G. and Biddle, F. (1972) Glycoproteins as influenza virus haemagglutinin inhibitors and as cellular virus receptors. In: A. Gottschalk (Ed), Glycoproteins, pp. 1082–1096. Elsevier, Amsterdam.

Grunert, R.R., McGahen, J.W. and Davies, W.L. (1965) The in vivo antiviral activity of 1-adamantamine (amantadine). I. Prophylactic and therapeutic activity against influenza viruses. Virology 26, 262–269.

Iinuma, M., Tanaka, T., Iwashima, K. and Matsuura, S. (1984) Synthesis of flavones by the use of isopropyl as a protective group. Yakugaku Zasshi 104, 691–694.

Lin, W., Oishi, K. and Aida, K. (1977) Specific inhibition of viral neuraminidase by an inhibitor, neuraminin, produced by *Streptomyces* sp. Virology 78, 108–114.

Meindl, P., Bodo, G., Palese, P., Schulman, J. and Tuppy, H. (1974) Inhibition of neuraminidase activity by derivatives of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid. Virology 58, 457–463.

Miyaichi, Y., Imoto, Y., Saida, H. and Tomimori, T. (1988) Studies on the constituents *Scutellaria* species (X). On the flavonoid constituents of the leaves of *Scutellaria baicalensis* Georgi. Shoyakugaku Zasshi 42, 216–219.

Morita, N. (1960) Studies on the flavonoid of sesame leaves. II. Synthesis of demethylpedalitin. Chem. Pharm. Bull. 8, 66–71.

Murti, K.G. and Webster, R.G. (1986) Distribution of hemagglutinin and neuramindase on influenza virions as revealed by immunoelectron microscopy. Virology 149, 36–43.

Nagai, T. and Yamada, H. (1988) Characterization of mouse liver sialidase and partial purification of the lysosomal sialidase. Chem. Pharm. Bull. 36, 4008–4018.

Nagai, T. Miyaichi, Y., Tomimori, T. and Yamada, H. (1989) Inhibition of mouse liver sialidase by plant flavonoids. Biochem. Biophys. Res. Commun. 163, 25–31.

Nagai, T., Miyaichi, Y., Tomimori, T., Suzuki, Y. and Yamada, H. (1990) Inhibition of influenza virus sialidase and anti-influenza virus activity by plant flavonoids. Chem. Pharm. Bull. 38, 1329–1332.

Palese, P., Schulman, J.L., Godo, G. and Meindl, P. (1974) Inhibition of influenza and parainfluenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA). Virology 59, 490–498.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and DeClercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Reutter, W., Köttgen, E., Bauer, C. and Gerok, W. (1982) Biological significance of sialic acids. In: R. Schauer (Ed), Cell Biology Monographs, Vol. 10, pp. 263–305. Springer, Vienna.

Schauer, R. (1982) Chemistry, metabolism, and biological functions of sialic acids. Adv. Carbohydr. Chem. Biochem. 40, 131–234.

Suzuki, Y., Sato, K., Kiso, M. and Hasegawa, A. (1990) New ganglioside analogs that inhibit influenza virus sialidase. Glycoconjugate J. 7, 349–356.

Tomimori, T., Miyaichi, Y. and Kizu, H. (1982) On the flavonoid constituents from the roots of *Scutellaria baicalensis* Georgi. I. Yakugaku Zasshi 102, 388–391.

Umezawa, H., Aoyagi, T., Komiyama, T., Morishima, H., Hamada, M. and Takeuchi, T. (1974) Purification and characterization of a sialidase inhibitor, siastatin, produced by *Streptomyces*. J. Antibiotics 27, 963–969.

Veh, R.W. and Schauer, R. (1978) Interaction of human brain neuraminidase with tritium-labelled gangliosides. In: S. Gatt, L. Freysz and P. Mandel (Eds), Adv. Exp. Med. Biol., Vol. 101, pp. 447–462. Plenum Press, New York.

Webster, R.G. and Laver, W.G. (1975) Antigenic variation of influenza viruses. In: E.D. Kilboune (Ed), The Influenza Viruses and Influenza, pp. 269–314. Academic Press, New York.

Wieley, D.C. and Skehel, J.J. (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. 56, 365–394.