Inhibition of Influenza Virus Sialidase and Anti-influenza Virus Activity by Plant Flavonoids

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Flavonoids (103 species) were tested for inhibitory activity against influenza virus sialidase using sodium *p*-nitrophenyl-*N*-acetyl-α-D-neuraminate as substrate. 5,7,4′-Trihydroxy-8-methoxyflavone from the root of *Scutellaria baicalensis* showed the most potent activity (IC_{50}, 55 μM), and this flavone appeared to be a non-competitive inhibitor of the enzyme. Whereas, negligible or weak inhibitory activities were observed for mouse liver sialidase, β-galactosidase and α-mannosidase as tested. This flavone also inhibited the infection by influenza virus A/PR/8/34 of Madin-Darby canine kidney cells, and replication of the virus in the allantoic sack of embryonated egg. These results suggest that flavone, which has potent influenza virus sialidase inhibitory activity, may have anti-influenza virus activity.

Keywords sialidase; neuraminidase; influenza virus; inhibitor; flavonoid; infection

Influenza virus express two envelope glycoproteins: hemagglutinin and sialidase [neuraminidase, EC 3.2.1.18]. The hemagglutinin is known to mediate the binding of viruses to target cells *via* sialic acid residue in glycoconjugates. This binding is a key step of the viral infection.[1] While, the sialidase presumably aids in the elution of newly formed viruses from the infected cells by digesting sialic acid from hemagglutinin receptor, and in the destruction of sialic acid containing mucus glycoproteins that can act as receptor analogs located on the host cell surface and inhibit infection.[1,2] Therefore, the inhibitors of influenza virus sialidase have the possibility of blocking infection by the influenza virus. Some plant flavonoids from *Scutellaria baicalensis* were shown to have significant inhibitory activity against mouse liver sialidase.[3] Therefore, the inhibitory activity of the flavonoids against influenza virus sialidase was tested. The present paper describes the inhibitory activity of flavonoids against influenza virus sialidase, and anti-influenza virus activity by the sialidase inhibitory flavonoids.

Materials and Methods

Materials Flavonoids were isolated or prepared as described previously.[4] 2,3-Dehydro-2-deoxy-*N*-acetylneuraminic acid (NeuAc2en) and sodium *p*-nitrophenyl-*N*-acetyl-α-D-neuraminate (PNP-NeuAc) were purchased from Boehringer Mannheim GmbH (Mannheim, West Germany) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Influenza HA vaccine and influenza virus A/PR/8/34 (H1N1) were obtained from the Kitasato Institute (Tokyo, Japan). Mouse liver sialidase was prepared as previously described.[5] Other PNP-glycopyranosides, jack bean α-mannosidase, Brewer's yeast α-glucosidase and almond β-glucosidase were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Jack bean β-galactosidase was from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan).

Sialidase Assay Flavonoids were dissolved in 50% dimethyl sulfoxide. Influenza virus sialidase activity was assayed in 0.11 ml of 25 mM citrate–phosphate buffer, pH 5.0, containing 25 nmol of PNP-NeuAc, flavonoid solution (10 μl), and influenza HA vaccine (10 μl) as the enzyme, then the reaction mixture was incubated at 37 °C for 15 min in a microtiter plate. The reaction was stopped by the addition of 0.19 ml of 0.2 M sodium borate buffer, pH 9.8. The *p*-nitrophenol liberated was determined from the absorbance at 405 nm with a Micro Plate Reader Model MPR-A4 (Tosoh). The percent inhibition of the sialidase activity was calculated as follows: % inhibition $=[(A-B)-(C-D)/A-B]\times 100$. *A*: Absorbance at 405 nm without test sample and with enzyme. *B*: Absorbance at 405 nm without test sample and enzyme. *C*: Absorbance at 405 nm with test sample and enzyme. *D*: Absorbance at 405 nm with test sample and without enzyme.

Mouse liver sialidase activity was assayed as previously described.[3b]

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.[3b]

***In Vitro* Anti-influenza Virus Activity** Influenza virus A/PR/8/34 (5.3×10^4 plaque forming units (PFU)) were inoculated into monolayers of Madin-Darby canine kidney (MDCK) cells (7.5×10^4 cells) in 0.25 ml of minimal essential medium containing 3 μg/ml of trypsin, and then the cells were incubated for 30 min at 34 °C in culture plates. The paper discs (thick type, 8 mm diameter) which absorbed flavonoid samples were put into the well of a culture plate, and the plate was cultured at 34 °C for 3 d under a 5% CO_2 atmosphere. The monolayers were washed with phosphate-buffered saline, pH 7.2 (PBS) to remove the dead cells, caused by infection of the influenza virus, then the living cells were stained with Giemsa solution. Anti-influenza virus activity was estimated by the stained area in the well bottom.

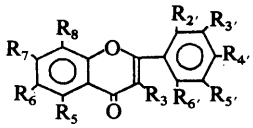
***In Vivo* Anti-influenza Virus Activity** The mixture of 0.2 ml of virus suspension (7×10^3 PFU/ml) and 0.1 ml of flavonoid solution was injected into the allantoic sack of embryonated eggs (10—11 d old), and then the eggs were incubated at 34 °C for 2 d. Allantoic fluid was harvested, and the amount of virus in the fluid was determined by hemagglutination assay using chicken erythrocytes and by sialidase activity with PNP-NeuAc.

Hemagglutination Assay Serial two-fold dilution of the virus (25 μl) was made in PBS containing bovine serum albumin (2 mg/ml) and 25 μl of 0.5% (v/v) chicken erythrocytes (in PBS) was added in microtiter V plate, mixed and the cells allowed to settle. Titration endopoints were determined after 60 min at room temperature.

Results and Discussion

A variety of flavonoids (80 kinds of flavones, 20 kinds of flavanones and 3 kinds of chalcones) were tested for their inhibitory effect on influenza virus sialidase activity. At final concentration of 91 μg/ml, only two kinds of flavones, F27 (340 μM) and F36 (300 μM), inhibited more than 50% of the enzyme activity (Table I). However, mouse liver sialidase was inhibited weakly by these flavones (Tables II and III). The flavanones[4a-c,e,f,h-j] and chalcones[4f,j] tested have little or no inhibitory activity against the influenza virus enzyme (data not shown). These results indicate that a certain flavone structure is required to inhibit influenza virus sialidase, and that inhibitors of influenza virus sialidase are different flavones from those of the mouse liver enzyme. The most potent inhibitor of influenza virus sialidase, 5,7,4′-trihydroxy-8-methoxyflavone (F36) from the root of *S. baicalensis*, inhibited the enzyme in a dose-dependent manner (Fig. 1A), and 50% of the influenza virus sialidase activity was inhibited in the presence of 55 μM of F36 (IC_{50}). This activity was 4 times more potent than a known sialidase inhibitor, NeuAc2en (IC_{50}, 220 μM). Apigenin (5,7,4′-trihydroxyflavone, F27) from the whole

TABLE I. Inhibitory Activity of Flavones on Influenza Virus Sialidase



No.	Substituent										Inhibition[a] (%)	Reference and note
	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$		
F1	H	OH	H	OH	H	H	H	H	H	H	0	4*a*
F2	H	OH	H	OGlcA	H	H	H	H	H	H	1.9	4*b*
F3	H	OiPr	H	OiPr	H	H	H	H	H	H	7.6	4*c*
F4	H	OH	H	OBz	H	H	H	H	H	H	6.0	4*c*
F5	H	OH	OH	OH	H	H	H	H	H	H	21.2	4*c*
F6	H	OAc	OAc	OAc	H	H	H	H	H	H	5.8	[b]
F7	H	OH	OMe	OH	H	H	H	H	H	H	5.0	4*c*
F8	H	OH	OMe	OAc	H	H	H	H	H	H	0	[c]
F9	H	OH	OMe	OMe	H	H	H	H	H	H	0	4*d*
F10	H	OH	OH	OGlcA	H	H	H	H	H	H	6.4	4*c*
F11	H	OH	OH	OGlcAMe	H	H	H	H	H	H	0	4*c*
F12	H	OH	OH	OGlc	H	H	H	H	H	H	9.5	4*c*
F13	H	OH	OMe	OGlcA	H	H	H	H	H	H	9.1	4*c*
F14	H	OH	OMe	OGlcAMe	H	H	H	H	H	H	0.5	4*c*
F15	H	OH	OH	Ogent	H	H	H	H	H	H	8.4	4*d*
F16	H	OH	H	OH	OH	H	H	H	H	H	28.7	4*c*
F17	H	OH	H	OH	OMe	H	H	H	H	H	4.1	4*c*
F18	H	OH	H	OBz	OH	H	H	H	H	H	6.7	4*c*
F19	H	OH	H	OMe	OMe	H	H	H	H	H	2.7	4*c*
F20	H	OMe	H	OH	OMe	H	H	H	H	H	0	4*c*
F21	H	OMe	H	OMe	OMe	H	H	H	H	H	0	4*c*
F22	H	OH	H	OGlcA	OMe	H	H	H	H	H	2.0	4*c*
F23	H	OMe	H	OGlcA	OMe	H	H	H	H	H	14.5	[d]
F24	H	OH	H	OAc	OMe	H	H	H	H	H	9.4	4*c*
F25	H	OMe	H	OAc	OMe	H	H	H	H	H	17.5	4*c*
F26	H	OH	H	OH	H	OH	H	H	H	H	0	4*c*
F27	H	OH	H	OH	H	H	H	OH	H	H	56.2	4*c*
F28	H	OH	H	OGlc–Rha	H	H	H	OH	H	H	3.6	4*c*
F29	H	OH	H	OGlc–Rha	H	H	H	OMe	H	H	5.1	[e]
F30	OGal–Glc	OH	H	OH	H	H	H	OH	H	H	28.0	4*c*
F31	H	OH	OH	OH	H	H	H	OH	H	H	0.7	4*b*
F32	H	OH	OMe	OH	H	H	H	OH	H	H	0	4*a*
F33	H	OH	OMe	OMe	H	H	H	OH	H	H	0.1	4*c*
F34	H	OH	OMe	OMe	H	H	H	OMe	H	H	8.2	4*b*
F35	H	OH	H	OH	OGlcA	H	H	OH	H	H	23.6	4*b*
F36	H	OH	H	OH	OMe	H	H	OH	H	H	53.2	4*c*
F37	H	OH	OMe	OMe	OH	H	H	H	H	H	1.5	4*c*
F38	H	OGlc	H	OH	H	H	OH	OH	H	H	9.3	4*c*
F39	H	OH	H	OGlc	H	H	OH	OH	H	H	6.4	4*c*
F40	H	OH	H	OH	H	OH	OH	H	H	H	4.8	4*c*
F41	H	OH	H	OH	OMe	OH	H	H	H	H	0	4*c*
F42	H	OH	H	OMe	OMe	OH	H	H	H	H	14.2	4*c*
F43	H	OH	H	OH	OMe	OMe	H	H	H	H	10.6	4*c*
F44	H	OH	H	OGlcA	OMe	OMe	H	H	H	H	0	4*e*
F45	H	OH	H	OMe	OMe	OAc	H	H	H	H	7.9	4*c*
F46	H	OMe	H	OMe	OMe	OMe	H	H	H	H	4.3	4*f*
F47	H	OAc	H	OMe	OMe	OAc	H	H	H	H	13.8	4*c*
F48	H	OH	OMe	OMe	OMe	OH	H	H	H	H	10.1	4*c*
F49	H	OH	OMe	OH	H	H	OH	OH	H	H	1.6	[f]
F50	H	OH	OMe	OMe	H	H	OMe	OH	H	H	0	4*a*
F51	H	OH	H	OH	OMe	OH	H	H	H	OMe	3.5	4*c*
F52	H	OH	H	OH	OMe	OMe	H	H	H	OMe	14.2	4*f*
F53	H	OH	H	OMe	OMe	OH	H	H	H	OMe	6.9	4*e*
F54	H	OH	H	OMe	OMe	OMe	H	H	H	OMe	13.8	4*f*
F55	H	OH	H	OMe	OMe	OMe	H	H	H	OBz	8.2	4*c*
F56	OH	OH	H	OH	H	OH	H	H	H	OH	0	4*c*
F57	OMe	OH	H	OMe	H	H	OMe	OMe	H	H	4.6	4*c*
F58	ORha	OH	H	OH	H	H	OH	OH	H	H	11.3	4*c*
F59	H	OH	OMe	OMe	OMe	OH	H	H	H	OMe	1.0	4*c*
F60	H	OH	OMe	OMe	OMe	OMe	H	H	H	OMe	6.3	4*c*
F61	H	OH	H	OH	OMe	OH	H	H	OH	OMe	4.8	4*c*
F62	H	OH	Glc	OH	H	H	H	OH	H	H	0	4*c*

TABLE I. (continued)

No.	Substituent										Inhibition[a] (%)	Reference and note
	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$		
F63	H	OH	Glc	OMe	H	H	H	OH	H	H	7.5	4c
F64	H	OH	Glc	OGlc	H	H	H	OH	H	H	2.2	4c
F65	H	OH	Glc	OMe	H	H	H	OMe	H	H	0	4c
F66	H	OMe	Glc	OMe	H	H	H	OMe	H	H	3.2	4c
F67	H	OAc	$GlcAc_4$	OMe	H	H	H	OAc	H	H	0	4c
F68	H	OH	Glc	OMe	H	H	H	OGlc	H	H	0	4g
F69	H	OH	Glc	OH	H	H	OH	OH	H	H	15.9	4c
F70	H	OH	Glc	OMe	H	H	OH	OH	H	H	6.2	4c
F71	H	OH	Glc–Rha	OMe	H	H	H	OMe	H	H	0	4c
F72	H	OH	H	OH	Glc	H	H	OH	H	H	17.5	4c
F73	H	OH	H	OMe	Glc	H	H	OH	H	H	0	4c
F74	H	OH	H	OMe	Glc	H	H	OMe	H	H	7.2	4c
F75	H	OMe	H	OMe	Glc	H	H	OMe	H	H	2.5	4c
F76	H	OH	H	OMe	C_5H_9	H	H	OH	H	H	0	g)
F77	OH	OH	H	OH	C_5H_9	H	H	OMe	H	H	8.8	4c
F78	ORha	OH	H	OGlc	C_5H_9	H	H	OH	H	H	18.1	4c
F79	ORha	OH	H	OGlc	C_5H_9	H	H	OMe	H	H	15.5	4c
F80	OH	OH	H	$-OC(CH_3)_2(CH_2)_2-$		H	H	OMe	H	H	0	4c

Abbreviations: Ac, acetyl; Bz, benzyl; C_5H_9, prenyl group; Gal, galactose; gent, gentiobiose; Glc, glucose; GlcA, glucuronic acid; iPr, isopropyl; Rha, rhamnose. *a*) Concentration of flavones; 91 μg/ml. *b*) Prepared by acetylation of F5. *c*) Prepared by partial acetylation of F7. *d*) Isolated from *Scutellaria rivularis* (unpublished). *e*) Isolated from *Exacum walkeri* (unpublished). *f*) Isolated from *Helenium autumnale* (unpublished). *g*) Isolated from *Dolabella auricularia* (unpublished).

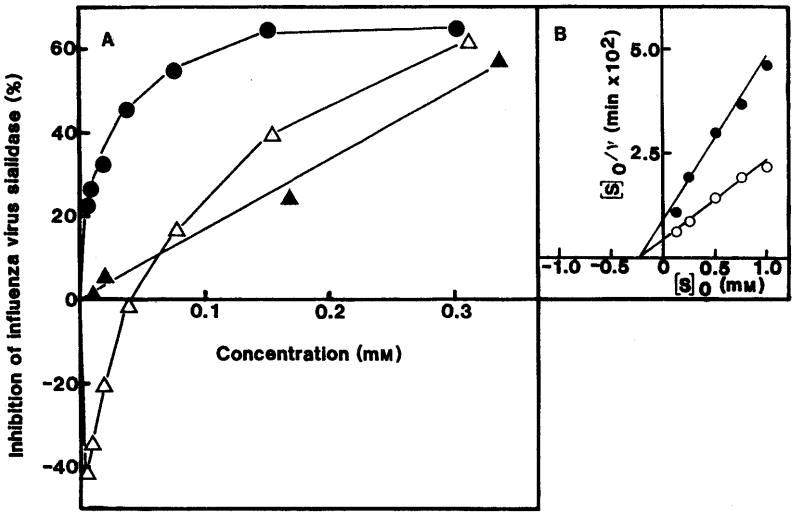


Fig. 1. Inhibitory Activity of 5,7,4′-Trihydroxy-8-methoxyflavone (F36) against Influenza Virus Sialidase

(A) Sialidase inhibitory activities of F27 (▲) and F36 (●) were compared with that of NeuAc2en (△) under the condition described in Materials and Methods. (B) Initial velocities of influenza virus sialidase activity were determined in the absence (○) or presence of F36 at 76 μM (●) in increasing PNP-NeuAc concentration, then $[S]_0/v$ were plotted against $[S]_0$.

herb *S. rivularis* inhibited the sialidase at 340 μM, but was less active at a lower concentration. The initial velocity (v) of influenza virus sialidase activity was measured at increasing PNP-NeuAc concentration in the presence or absence of 76 μM of F36, then the initial concentrations of PNP-NeuAc ($[S]_0$) were plotted against $[S]_0/v$. As seen in Fig. 1B, F36 decreased the maximal velocity (V_{max}) of influenza virus sialidase two-fold (5.4 to 2.7 μM/min), but did not significantly alter the K_m value (0.24 mM) of the enzyme for PNP-NeuAc. Therefore, F36 appeared to be acting as a non-competitive inhibitor of influenza virus sialidase. The inhibition constant (K_i value) of F36 calculated using the values of V_{max} obtained at 0 and 76 μM in this assay was 77 μM. The relationship between the structure of flavones and sialidase inhibitory activity was not clear, but trihydroxylated flavones have a relatively potent inhibitory activity (F5, F16, F27, F30, F35 and F36) (Table I). F36 (76 μM) showed little or no inhibition of jack bean β-galactosidase and jack bean α-mannosidase, but same concentrations of F36 inhibited other glycosidases, such as yeast α-glucosidase and almond β-glucosidase, less than influenza virus sialidase (Table II).

Flavones which have potent inhibitory activity against influenza virus or mouse liver sialidases were tested for anti-influenza virus activity *in vitro* and *in vivo*. F36, which is a most potent influenza virus sialidase inhibitor, inhibited the infection by influenza virus A/PR/8/34 of MDCK cells significantly, even when 10 μg/ml (33 μM) of a sample was used (Table III). F36 also inhibited replication of the virus in the allantoic sack of embryonated egg at 100 μg

Table II. Inhibitory Activity of F36 against Various Glycosidases

Glycosidase	Concentration (μM)[a]	Inhibition (%)
Sialidase (influenza virus)	76	54.7
Sialidase (mouse liver)	76	12.5
	159	29.5
β-Galactosidase (jack bean)	76	7.0
α-Mannosidase (jack bean)	76	0
α-Glucosidase (yeast)	76	27.8
β-Glucosidase (almond)	76	29.1

a) 76 μM is 23 μg/ml and 159 μM is 48 μg/ml, respectively.

Table III. Relationship between Sialidase Inhibitory Activity and Anti-influenza Virus Activity (*in Vitro*) of Flavones

Flavone	Sialidase inhibitory activity (%)		Anti-virus activity (%)[a]		
	Mouse liver[b]	Influenza virus[c]	1000 μg/ml	100 μg/ml	10 μg/ml
F16	1.5	28.7	n.d.	D	D
F17	13.6	4.1	C	B	C
F27	17.9	56.2	n.d.	A—B	C
F35	100	23.6	n.d.	D	C
F36	29.5	53.2	B—C	B—C	B—C
F46	63.5	4.3	D	n.d.	n.d.
F57	54.0	4.6	D	n.d.	n.d.

a) Anti-influenza virus activity was expressed as % survival of the cells: A, 100—90%; B, 80—40%; C, 30—10%; D, 0%, and flavones showed no toxicity against MDCK cells. *b*) Concentration; 48 μg/ml (see Ref. 3*b*). *c*) Concentration; 91 μg/ml. n.d., not determined.

(0.33 μmol)/egg completely, because negligible hemagglutination titer and sialidase activity, which are caused by residual virus, were observed in the allantoic fluid at 2 d after application of virus (Table IV). These results suggest that flavone which has potent influenza virus sialidase inhibitory activity may have anti-influenza virus activity, and measurement of influenza virus sialidase inhibitory activity is useful for the screening of anti-influenza virus substances. F27 (100 μg/ml, 370 μM) showed significant anti-influenza virus activity *in vitro* (Table III), but no activity *in vivo* (Table IV). This result may be caused by the dilution of F27 in the egg, because F27 showed only weak influenza virus sialidase inhibitory activity at a low concentration (Fig. 1A). It was reported that an extract of the root of *S. baicalensis* inhibited infection by the influenza virus A/PR/8 in mice.[6] F36 may be an active substance in this anti-virus activity, because F36 was purified from the root of *S. baicalensis*.[4c] NeuAc2en also inhibited replication of influenza virus in embryonated eggs (Table IV). But NeuAc2en and other known sialidase inhibitors inhibit not only influenza virus sialidase but also mammalian sialidases.[3b,7] However, F36 shows a stronger effect as an influenza virus sialidase inhibitor than for mouse liver enzyme (Table II). These results indicate that F36 should prove to be a useful and specific anti-influenza virus substance by inhibiting the virus sialidase.

Table IV. Anti-influenza Virus Activity (*in Vivo*) of Flavones

Compound[a]	Hemagglutination titer (units/ml)	Sialidase activity (munits/ml)[b]
No addition	1280	66.1
F17	640	69.8
F27	640	59.8
F36	0	0.5
NeuAc2en	0	0.1

a) Dose; 100 μg/egg. *b*) One unit was defined as the amount of enzyme which hydrolyzed 1 μmol of PNP-NeuAc/min.

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