***In Vitro* Antiallergic Activity of Flavonoids in Histamine Release Assay Using Rat Basophilic Leukemia (RBL-2H3) Cells**

(Received April 15, 1994)

Masaru KAWASAKI^{*1, *2}, Masatake TOYODA^{*1}, Reiko TESHIMA^{*1},
Jun-ichi SAWADA^{*1}, Toshimitsu HAYASHI^{*3}, Munehisa ARISAWA^{*3},
Mineo SHIMIZU^{*3}, Naokata MORITA^{*3}, Syozo INOUE^{*4}
and Yukio SAITO^{*1}

(^{*1}National Institute of Health Sciences: 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan;

^{*2}Present address: Hatano Research Institute, Food and Drug Safety Center: 729-5, Ochiai, Hadano-shi, Kanagawa 257, Japan, To whom reprint requests should be addressed; ^{*3}Faculty of Pharmaceutical Sciences, Toyama Medical &

Pharmaceutical University: 2630, Sugitani, Toyama 930-01, Japan;

^{*4}Nitto Denko Co.: 1-1-2, Shimohozumi, Ibaraki, Osaka 567, Japan)

We used an established cell line, rat basophilic leukemia cells (RBL-2H3) to screen 40 flavonoids for inhibitory activity on antigen-induced histamine release from immunoglobulin E (IgE)-sensitized RBL-2H3 cells. To exclude non-specific inhibition, the cytotoxicity to RBL-2H3 cells was simultaneously determined. Flavonoid aglycones showed a stronger activity for histamine release-inhibition and cytotoxicity than glycosides, and both activities were almost in parallel. Baicalein showed histamine release-inhibitory activity with the IC_{50} of 1.07×10^{-5} M in this bioassay system. However, it showed a potent cytotoxicity (IC_{50} 9.62×10^{-6} M). On the other hand, scutellarein (4'-hydroxybaicalein) showed a potent histamine release-inhibitory activity (IC_{50} 3.15×10^{-6} M) and low cytotoxicity (IC_{50} 6.11×10^{-5} M). We found that scutellarein has a potent histamine release-inhibitory activity and low cytotoxicity.

Key words: rat basophilic leukemia cell; antiallergy; histamine release-inhibitor; cytotoxicity; flavonoid; scutellarein

Introduction

Flavonoids, a large group of naturally occurring pigments, are widely distributed in the plant kingdom and also are major components in some edible plants. Certain flavonoids inhibit various enzymes, such as phosphodiesterase³⁻⁵⁾, Ca^{2+} -ATPase⁶⁾, aldose reductase⁷⁻⁹⁾, lipoxygenase^{10, 11)}, cyclooxygenase and xanthine oxidase¹²⁾. Several flavonoids have a histamine release-inhibitory activity on rat peritoneal mast cells¹³⁻¹⁵⁾. However, comparative studies on the antiallergic activity of many kinds of flavonoids have not yet been done. The release of inflammatory mediators, such as histamine and leukotrienes, from mast cell or basophils, is med-

iated by cell-surface receptors for immunoglobulin E (IgE), and stimulation by antigen results in allergic reactions called immediate-type hypersensitivity. *In vitro* antiallergic screening has generally been performed by using rat peritoneal mast cells, but a disadvantage of this method is that mast cells must be prepared from experimental animals before each assay. Rat basophilic leukemia cells (RBL-2H3) release histamine, serotonin and β -hexosaminidase when exposed to various stimuli *in vitro*¹⁾. The antigen-induced histamine release from IgE-sensitized RBL-2H3 cells provides an *in vitro* model for *in vivo* antigen-induced allergic reactions. Since RBL-2H3 is an established cell line and easy to subculture, antiallergic screening

assay using this cell line should be rapid and reproducible. In this paper we describe a bioassay system using RBL-2H3 cells to screen histamine-release inhibitors among 40 flavonoids. Cytotoxic activity was simultaneously determined to find substances which show a strong inhibitory effect on histamine release from RBL-2H3 cells combined with low cytotoxicity.

Materials and Methods

Cells and medium

RBL-2H3 cells (JCRB 0023) were obtained from the Japan Cancer Research Resources Bank. The medium used for cell culture was Eagle's minimum essential medium (MEM; Kohjin Bio Co. Ltd.) supplemented with 10% fetal calf serum (FCS, GIBCO Co. Ltd.) and 0.08 mg/ml kanamycin (Nakarai Co.). Monoclonal IgE antibody-producing hybridoma (IGELa2) was purchased from ATCC (TIB 142).

Buffer

The following buffer was used: 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1.0 mM CaCl₂, 5.5 mM glucose, 0.1% bovine serum albumin (BSA), and 5 mM *n*-2-(hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4.

Antigen (DNP-BSA) and monoclonal IgE antibody

Dinitrophenylated BSA was prepared by dinitrophenylation of BSA with 2,4-dinitrobenzenesulfonic acid. The extent of dinitrophenylation was 7 mol per mole of BSA. Monoclonal mouse IgE antibody specific for the 2,4-dinitrophenyl group was prepared by using IGELa2¹⁶⁾ cells. The cells were propagated *in vivo* in pristane-treated BALB/c mice, and the ascites was used as anti-TNP IgE.

Flavonoids

We isolated and identified most of the flavonoids from plant sources. Most of the flavonoids listed in Table 2 were the same samples as those reported by Hayashi *et al.*¹²⁾ All flavonoids were dissolved in DMSO and the solution was diluted in medium, so that the concentration of DMSO in the medium was under 0.2%. This concentration of DMSO did not interfere with the bioassay or analysis of histamine.

Measurement of histamine release-inhibitory activity

Measurement of histamine release-inhibitory activity was performed by the method of Teshima *et al.*²⁾ with a slight modification. RBL-2H3 cells (1×10^5 cells/well) were plated in 24-well flat-bottomed microtiter plates (Costar 3424) in 0.5 ml per well of MEM containing 10% FCS and incubated for 24 hr at 37°C. The supernatants were discarded, and the cells were incubated at 37°C for 120 min with 0.5 ml of MEM containing 2% FCS and TNP-specific monoclonal IgE antibody (1000-fold-diluted ascites). Then the supernatants were removed and the cells were washed three times with the buffer to remove unbound IgE. After incubation at 37°C for 10 min with a flavonoid-containing buffer (0.4 ml), the cells were challenged with 100 μ l of DNP-BSA (100 μ g/ml of the buffer) for 35 min at 37°C. Cold HEPES buffer (600 μ l) was added to stop the reaction. One ml of the supernatant was withdrawn from each well and mixed with 20 μ l of perchloric acid. The supernatant was centrifuged at 12,000 rpm for 30 min at 2°C and then filtered through a 2 μ m Millipore filter. The filtrates were submitted to HPLC. Histamine release-inhibitory activities (% inhibition) were calculated as follows: % Inhibition = $100 \times \{1 - [(SR-SC)/(T-SC)]/[(R-C)/(T-C)]\}$, where T is the mean of total histamine content determined by adding perchloric acid (2% final concentration) to the same sample; C is the histamine content spontaneously released from control cells; SC is the histamine content spontaneously released from flavonoid-treated cells; R is the histamine content released from control cells by antigen stimulation; SR is the histamine content released from flavonoid-treated cells by antigen stimulation. The IC₅₀ was determined as the 50%-inhibitory concentration compared to the control by plotting the inhibition data as dose-effect curves (Fig. 2), from which the 50%-inhibitory concentration was calculated.

Measurement of histamine content of medium

Released histamine was measured by a Shimadzu histamine analysis system based on the HPLC method of Somehara *et al.*,¹⁷⁾ using post column derivatization with *ortho*-phthalaldehyde (OPA). Figure 1 shows a schematic flow

diagram of the system and analytical conditions.

Cytotoxicity

RBL-2H3 cells were plated in 24-well flat-

bottomed microtiter plates (Costar 3424) in 0.5 ml of MEM containing 10% FCS per well and incubated for 72 hr at 37°C in the presence of flavonoid. The medium was removed, the cell monolayers were trypsinized, and the viable cell

Apparatus: 1, 2, 3; pumps, 4; injector, 5; oven, 6; detector, 7; data processor, 8; reactor, 9; valve, 10; valve, 11; analytical column, 12; trap column, 13; pre-column, 15, 16, 17; mobile phase, 18; reagent

Analytical conditions

[For separation]

Column	Shim-pack PAG-SP (10 mm×8 mm ϕ) for pretreatment Shim-pack ISC-05 (10 mm×4 mm ϕ) for trapping Shim-pack ISC-05 (38 mm×4.6 mm ϕ) for separation
Mobile phase	100 mM (sodium) EDTA <pH=6.0> containing 10 mM sodium dodecylsulfate for pretreatment 100 mM (sodium) EDTA <pH=8.5> containing 1 M sodium perchlorate for separation 100 mM (sodium) EDTA <pH=8.5> for cleaning
Flow rate	0.6 ml/min
Temperature	50°C
Programming	trap column into analytical line at 3.3 min trap column into pretreatment line at 7 min

[For detection]

Reagent	1% OPA acetonitrile solution and 4 mM sodium dihydrogenphosphate dihydrate <pH=2.8> aqueous solution (1 : 20 v/v)
Flow rate	0.3 ml/min
Reactor	piping part J (400 μ l)
Temperature	50°C
Detection	fluorescence at $Ex=360$ nm; $Em=440$ nm

[Sample]

Injection volume 100 μ l

Fig. 1. Schematic flow diagram of the Shimadzu histamine analysis system and analytical conditions

Fig. 2. Cytotoxicity and inhibitory effects of scutellarein and baicalein on histamine release from RBL-2H3 cells

number was determined by a trypan blue dye exclusion method. Cytotoxicity (% inhibition) was calculated as follows: % inhibition = $100 \times [1 - (\% \text{ viable cell in flavonoid-treated cells}) / (\% \text{ viable cell in drug-untreated cells})]$

Results and Discussion

Bio-assay

RBL-2H3 cells sensitized with anti-TNP IgE were preincubated with each test substance for 10 min. Then, the cells were stimulated by the antigen, DNP-BSA. In this assay, ketotifen fumarate salt, which is an antiallergic compound, showed a histamine release-inhibitory activity with an IC_{50} value of $8.46 \times 10^{-5} M$. It has been reported that RBL-2H3 cells have a similar function to that of mucosal-type mast cells as regards type I allergic reaction^{18, 19}.

Antiallergic effect and cytotoxicity of flavonoids

The inhibitory activities of 40 flavonoids against histamine release and their cytotoxicity at

Table 1. Histamine Release-Inhibitory Activity and Cytotoxicity of Flavonols and Their Derivatives

	Inhibition of histamine release		Cytotoxicity	
	Inhibition % (20 µg/ml)	$IC_{50}(M)$	Inhibition % (20 µg/ml)	$IC_{50}(M)$
Kaempferol	84.0	1.25×10^{-5}	66.0	—
Quercetin	84.3	2.05×10^{-5}	85.0	4.13×10^{-5}
Myricetin	59.9	—	16.7	—
3-O-Methyl quercetin	74.2	—	80.0	3.80×10^{-6}
3'-O-Methyl quercetin	65.2	—	70.0	6.30×10^{-5}
Quercetin 3-O-glucuronide	36.2	—	10.0	—
Quercetin 3-O-glucoside	5.2	—	29.6	—
Quercetin 3-O-rhamnoside	40.7	—	0.0	—
Quercetin 3-O-galactoside	61.6	—	25.0	—
Quercetin 3-O-arabinofuranoside	53.5	—	25.0	—
Quercetin 3-O-rutinoside	36.2	—	5.0	—
Quercetin 3-O-glucoside 2''-O-gallate	16.8	—	5.0	—
Quercetin 3'-O-methyl-4'-O-glucoside	24.3	—	81.5	1.40×10^{-6}
Myricetin 3-O-rhamnosylgalactoside	71.4	—	7.4	—
Myricetin 3-O-rhamnoside	51.6	—	0.0	—
Myricetin 3-O-arabinoside	36.0	—	3.7	—

—: Not tested

—: Not tested; R₁: Glucuronide; R₂: Glucoside; R₃: Rhamnosylglucoside

Table 3. Histamine Release-Inhibitory Activity and Cytotoxicity of C-Glycosylated Flavones and Their Derivatives

	Substituents					Inhibition of histamine release		Cytotoxicity	
	R ₆	R ₇	R ₈	R _{3'}	R _{4'}	Inhibition % (20 µg/ml)	IC ₅₀ (M)	Inhibition % (20 µg/ml)	IC ₅₀ (M)
Vitexin	H	OH	Glc-	H	OH	16.0	—	19.5	—
Swertisin	Glc-	MeO	H	H	OH	8.1	—	2.9	—
Embinin	Rhm-O-Glu-	OH	H	H	OMe	40.8	—	0.0	—
iso-Orientin	Glc-	OH	H	OH	OH	5.8	—	4.1	—
iso-Orientin 7-O-glucoside	Glc-	Glc-O-	H	OH	OH	30.0	—	29.5	—

—: Not tested; Glc: Glucose; Rhm: Rhamnose

have strong inhibitory activity on histamine release. Quercetin and myricetin glycosides did not show either activity. It seems that flavonols require a 3-OH group for inhibitory activity, and a 3-O-glycoside group may sterically hinder the active site of the aglycone.

Flavones

Histamine release-inhibitory activity and cytotoxicity of flavones are summarized in Table 2. Both apigenin and luteolin have histamine release-inhibitory activity and cytotoxicity. Baicalein, a well known antiallergic flavonoid^{14), 15)}, showed histamine release-inhibitory activity with the IC₅₀ of 1.07×10^{-5} M in this bio-assay system. However, it showed a potent cytotoxicity (IC₅₀ 9.62×10^{-6} M). On the other hand, scutellarein (4'-hydroxybaicalein) showed a potent histamine release-inhibitory activity (IC₅₀ 3.15×10^{-6} M) and low cytotoxicity (IC₅₀ 6.11×10^{-5} M) (Fig. 1). Glycosides of the 7-position of baicalein and scutellarein have low histamine release-inhibitory activity. Moreover, histamine release-inhibitory activity was observed in scutellarein 4'-methyl ether, pectrinarigenin, sorbifolin and cirsimaritin (Table 2).

Scutellarein (4'-OH) has a stronger inhibitory activity (more than one order) than baicalein (4'-H), but scutellarein 4'-methyl ether is about one order less active. A similar phenomenon was observed with pectrinaigenin (4'-OMe) vs.

sorbifolin (4'-OH). These findings suggest that a free hydroxyl group at the C-4' position in the B-ring increases the histamine release-inhibitory activity and substitution or absence of the free C-4' hydroxyl group decreases the activity. Flavonoids methoxylated (more than two) in the A-ring or glycosylated in the A-ring are less active. 5,6,7,8,4'-pentahydroxyflavone has a lower activity, possibly because this *para*-quinone-type flavonoid is easily decomposed.

Middleton *et al.*^{20), 21)} revealed critical structure-activity relationships governing the flavonoid effect on antigen-induced histamine release from human basophils. Inhibitory activity was associated with the presence of the C4-keto-5-OH couplet and was enhanced by the additional presence of the C3-OH group and an appropriate pattern of hydroxylation in the B-ring. Lim asset *et al.*²²⁾ have also examined the effect of flavonoids on the release of reactive oxygen species from stimulated human neutrophils. They affirmed the importance of A-ring (5-OH, 7-OH) and B-ring (3'-OH, 4'-OH) dihydroxylation and C-ring (3-OH) hydroxylation. This potency is generally decreased by glycosylation. Thus, 5-OH and free hydroxy groups in the A- or B-ring are important for activity. In our screening assay, all histamine release-inhibitory flavonoids had a free-OH group at the C-5 position. Histamine release-inhibitory activity of baicalein-type flavonoids required the aglycone, a free

5-hydroxyl group, and a 6- or 7-hydroxyl group, and furthermore, the presence of a 4'-hydroxyl group enhanced the activity.

C-Glycosylated flavone

C-Glycosylated flavonoids (listed in Table 3) were less active.

Acknowledgment

We are indebted to Dr. Yutaka Kikuchi, National Institute of Health Sciences, for suggesting the use of cultured RBL-2H3.

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