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STUDIES ON INHIBITORS OF SKIN TUMOR PROMOTION, III.[1] INHIBITORY EFFECTS OF ISOFLAVONOIDS FROM *WISTERIA BRACHYBOTRYS* ON EPSTEIN-BARR VIRUS ACTIVATION

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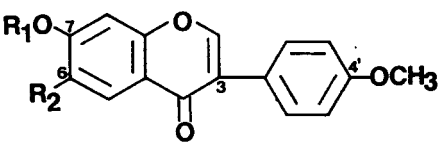
The knots of *Wisteria brachybotrys* Sieb. et Zucc. (Leguminosae) have been used in Japanese folk medicine for the treatment of gastric cancer (1). In a previous paper, we have reported that some triterpenes and triterpenoid saponins show significant inhibitory effects on the Epstein-Barr virus early antigen (EBV-EA) activation (2,3). As a result of our continuing search among medicinal plants for novel, naturally occurring, potential antitumor promoters, the MeOH extract of knots of *W. brachybotrys* was found to show significant inhibitory effects on EBV-EA activation in Raji cells (4). Many compounds that inhibit EBV-EA induction by tumor promoters have been shown to act as inhibitors of tumor promotion in vivo (5–8).

Furthermore, the tumor promoter 12-*O*-tetradeceanoylphorbol-13-acetate (TPA) was shown to enhance ^{32}P incorporation into cell phospholipids. It was also proved that this compound acts at early stages of tumor promotion, and it was suggested that it plays an important role in tumor promotion in vivo (9). Therefore, we examined the inhibitory effect of isoflavonoids from *W. brachybotrys* on EBV-EA activation and TPA-stimulated ^{32}P incorporation into phospholipids of HeLa cells (10).

Bioassay-directed fractionation of the active extract led to the isolation and characterization of afromosin [**1**], formononetin [**2**], wistin [**3**], and ononin [**4**] as inhibitory principles on EBV-EA activation. Then, to approach the inhibitory mechanism of isoflavonoids, some of them were also tested for their effect on binding to the TPA receptor in an epidermal particulate fraction by the cold Me_2CO filter method (11). Although these isofavonoids were previously isolated from *Wisteria floribunda* (12,13), their potent antitumor promoter activities are revealed here for the first time.

Four isoflavonoids **1**–**4**, their acetates **5** and **6**, and methyl ethers **7** and **8** were



	R_1	R_2
1	H	OMe
2	H	H
3	glucose	OMe
4	glucose	H
5	Ac	OMe
6	Ac	H
7	Me	OMe
8	Me	H

[1]For Part II, see Konoshima *et al.* (4).

TABLE 1. Inhibitory Effects of Isoflavonoids from *Wisteria brachybotrys* on Epstein-Barr Virus Early Antigen (EBV-EA) Activation.

Sample	% to control (% viability)[a]				EA activation [c]
	Concentration (mol ratio compound/TPA)				
	1×10^4	1×10^3	1×10^2	1×10	
1	0.0 + 0[b] (80.0)	36.4 + 8.1[b] (>80)	75.8 + 5.8[b] (>80)	100.0 + 0[b] (100)	34.0
2	0.0 + 0 (20.0)	63.6 + 5.4 (>80)	92.6 + 2.1 (>80)	100.0 + 0 (100)	0.0
3	15.2 + 10 (50.0)	78.5 + 7.2 (>80)	86.6 + 2.6 (>80)	100.0 + 0 (100)	0.0
4	39.3 + 8.5 (80.0)	76.0 + 6.6 (>80)	100.0 + 1.3 (100)	100.0 + 0 (100)	2.0
5	0.0 + 0 (80.0)	6.0 + 8.5 (>80)	42.3 + 5.8 (>80)	72.7 + 2.5 (100)	2.1
6	0.0 + 0 (80.0)	15.2 + 2.6 (>80)	95.2 + 0 (100)	100.0 + 0 (100)	0.0
7	0.0 + 0 (0.0)	66.8 + 3.5 (>80)	95.0 + 0 (100)	100.0 + 0 (100)	1.0
8	30.3 + 1.5 (80.0)	90.2 + 0 (100)	100.0 + 0 (100)	100.0 + 0 (100)	0.0

[a]In this screening method, the cell viability required for the judgment of inhibitory effects was more than 60% (14); 100 = positive control; TPA (32 pM). Each value represents the average of three determinations + SD.

[b]Each value in this column represents the % of EA activation by each compound alone (320 nM).

tested utilizing the short term in vitro assay on EBV-EA activation in Raji cells induced by TPA (2,14). Their inhibitory effects on activation and viabilities of Raji cells are shown in Table 1.

The isoflavonoids, **1**, 7-*O*-acetylafromosin [**5**], and 7-*O*-acetylformononetin [**6**], showed remarkable inhibitory effects on EBV-EA activation and preserved high viabilities of Raji cells even at high doses (1×10^4 mol ratio). Especially, **5** showed a significant inhibitory activity even at a low dose (60% inhibition of activation even at 1×10^2 mol ratio). On the other hand, 2,3,7-*O*-methylafromosin [**7**], and 7-*O*-methylformononetin [**8**] exhibited high cytotoxicity on Raji cells at 1×10^4 mol ratio. The inhibitory effects of the antipromoters retinoic and glycyrrhetinic acids have been reported by Tokuda *et al.* (8). From our experiments, it was deduced that the potency of the inhibitory activity of **5** was more than 10 times higher than those of retinoic and glycyrrhetinic acids, and that cytotoxicity of **5** on Raji cells was less than 100 times lower than that reported for both acids.

The effects of these isoflavonoids on TPA-stimulated ^{32}P incorporation into phospholipids of HeLa cells are shown in Table 2. At the concentration of 50 M, **1**, **2**, **5**, and **6** showed 16.4%, 34.5%, 33.9%, and 40.2% inhibition, respectively, of ^{32}P incorporation. Under the experimental conditions in this study, isoflavonoids did not show any cytotoxic effect on HeLa cells; the number of cells was not decreased, and viability of the cells was not affected by the treatment with these test compounds.

The present investigation by in vitro assays strongly suggested that compounds **5** and **6** may be valuable antipromoters and that the inhibitory effects of the extract of *W. brachybotrys* on EBV-EA activation is due to the combined effects of these two isoflavonoids.

From the results of a binding assay, it was deduced that **1**, **2**, and **5** showed no effect on [^3H]-TPA binding to the TPA receptor, while TPA and teleocidin B significantly inhibited it. These results indicated that isoflavonoids may act at some point after the binding of the tumor promoters to the receptors.

The details of the inhibitory mechanisms of isoflavonoids, the inhibitory effects of the combined isoflavonoids, and the initiation-promotion testings in vivo are now in progress.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Yanagimoto micro melting point apparatus and were uncorrected. ^1H- and ^{13}C-nmr spectra were recorded on a Varian XL-300 using TMS as an internal standard. Mass spectra were determined on a Hitachi M-80 mass spectrometer. Uv spectra were recorded on a Shimadzu UV-240 spectrometer in 95% EtOH. Si gel (Merck G 60, 230 mesh) was used for column and for flash chromatography (15), and Si gel plates (Merck, 60 F-254, 0.25

TABLE 2. Inhibitory Effects of Isoflavonoids from *Wisteria brachybotrys* on TPA-Enhanced ^{32}P Incorporation into Phospholipids of HeLa Cells at the Concentration of 50 μM.

Condition	^{32}P Incorporation (cpm/mg protein, $\times 10^{-4}$)	Inhibition (%)
Control	7.43	
+TPA	31.37	
+TPA + afromosin [**1**]	27.44	16.4
+TPA + formononetin [**2**]	23.11	34.5
+TPA + wistin [**3**]	33.12	0.0
+TPA + ononin [**4**]	28.43	12.3
+TPA + 7-*O*-acetylafromosin [**5**]	23.25	33.9
+TPA + 7-*O*-acetylformononetin [**6**]	21.75	40.2
+TPA + 7-*O*-methylafromosin [**7**]	25.94	22.7
+TPA + 7-*O*-methylformononetin [**8**]	23.16	34.3

TABLE 3. Inhibitory Effects of Extracts of *Wisteria brachybotrys* on Epstein-Barr Virus Early Antigen (EBV-EA) Activation.

Extract	% to control (% viability)[a]		
	Concentration (μg/ml)		
	100	10	1
MeOH	15.3 + 5.4 (60.0)	66.0 + 2.1 (>80)	100.0 + 0.0 (100)
n-Hexane	0.0 + 6.4 (60.0)	79.5 + 1.2 (>80)	98.8 + 0.6 (100)
$CHCl_3$	0.0 + 7.4 (10.0)	20.0 + 3.0 (70.0)	100.0 + 0.0 (100)
EtOAc	20.5 + 5.0 (10.0)	55.4 + 1.8 (70.0)	72.8 + 1.2 (80.0)
n-BuOH	42.3 + 2.3 (60.0)	80.6 + 0.9 (80.0)	100.0 + 0.0 (100)

[a]Each value represents the average of three determinations + SD.; 100 = positive control; TPA (20 ng/ml).

mm) were used for analytical tlc. Compounds were visualized by uv light and by spraying with 10% cerium (IV) sulfate in 10% H_2SO_4 solution followed by heating.

PLANT MATERIAL AND EXTRACTION.—The knots of *W. brachybotrys* were collected in Shikoku, Japan, in 1986. Herbarium specimens are deposited in the herbarium of Kyoto Pharmaceutical University. The chopped up knots of *W. brachybotrys* (2.5 kg) were exhaustively extracted with 80% hot MeOH. After the solvent was removed in vacuo, a dark brown syrup remained. Extraction and fractionation of the active compounds were monitored by an in vitro short-term assay of EBV-EA activation in Raji cells (2).

The active syrup was suspended in H_2O and extracted several times with *n*-hexane. The aqueous layer was extracted with $CHCl_3$, EtOAc, and *n*-BuOH saturated with H_2O, successively. Each organic layer was evaporated in vacuo to give residues (*n*-hexane 5 g, $CHCl_3$ 40 g, EtOAc 17 g, and *n*-BuOH 42 g). The results of the bioassay of these extracts are in Table 3.

ISOLATION OF COMPOUNDS **1**–**4**.—The $CHCl_3$ extract (3 g) was fractionated by cc on Si gel [solvent MeOH-$CHCl_3$ (1:9)] to afford 15 fractions. These fractions were subjected to the above-mentioned assay. Purification of the most active fractions by flash chromatography [solvent $CHCl_3$, pressure 1.8 kg/cm^2 (N_2 gas flow)] followed by recrystallization from MeOH yielded 280 mg of **1** and 85 mg of **2** as colorless needles.

The EtOAc extract (2 g) was also fractionated by cc on Si gel [$CHCl_3$-MeOH-H_2O (8:3:1)]. Purification of the most active fraction by flash chromatography [$CHCl_3$-MeOH (9:1)] followed by recrystallization from MeOH/H_2O yielded 80 mg of **3** as colorless needles and 30 mg of **4** as colorless prisms.

Identification of these compounds was achieved by comparison with the reported data (12,13).

ACETYLATION OF AFROMOSIN [**1**] AND FORMONONETIN [**2**].—Afromosin and formononetin were acetylated with Ac_2O in pyridine at room temperature for 18 h followed by usual workup and purification by recrystallization to yield 7-*O*-acetylafromosin [**5**] and 7-*O*-acetylformononetin [**6**], respectively. Identification of these compounds was achieved by comparison with the reported data (12,13).

METHYLATION OF **1** AND **2**.—Afromosin and formononetin were methylated with CH_2N_2 in Et_2O and MeOH followed by usual workup and purification by recrystallization to yield 7-*O*-methylafromosin [**7**] and 7-*O*-methylformononetin [**8**]. Identification of these compounds was achieved by comparison with the reported data (12,13).

BIOLOGICAL ACTIVITIES.—The assay of inhibitory effects of $CHCl_3$ extract and each pure compound on EBV-EA activation were carried out according to the short-term assay described previously (2,14).

Binding assay.—Specific [^{3}H]-TPA binding was assayed by the cold Me_2CO filter method. An epidermal particulate fraction was prepared from dorsal epidermis of female ICR mice. Protein (100 mg) from the particulate fraction was incubated at 4° for 3 h with [^{3}H]-TPA (4 nM) with the test compound in 1 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 2-mercaptoethanol (2 mM). Nonspecific binding was determined by measuring the binding of the [^{3}H]-TPA to the particulate fraction in the presence of a five hundred fold excess of unlabeled TPA.

Incorporation of ^{32}P into phospholipids of HeLa cells.—HeLa cells were maintained as monolayers in Eagle's minimum essential medium supplemented with 10% calf serum under a humidified atmosphere of 5% CO_2 in air. HeLa cells were inoculated into culture dishes (35 mm diameter), and, after 48 h, a test compound was

added to the culture medium. Incubation was continued for 1 h, and then the cells were labeled for 4 h with ^{32}P (10 Ci/culture) in the presence or absence of TPA (50 nM). The cells were harvested by trypsinization and were washed with phosphate-buffered saline (PBS). The cells were resuspended in PBS, and then total lipids were extracted by the method described previously (16). The radioactivity in aliquots of the phospholipid fraction was counted. Data are mean values of duplicate experiments and expressed as percent of inhibitory effect on TPA-stimulated ^{32}P incorporation.

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