Studies on Inhibitors of Skin Tumor Promotion. XI.[1] Inhibitory Effects of Flavonoids from *Scutellaria baicalensis* on Epstein-Barr Virus Activation and Their Anti-tumor-Promoting Activities[2,3]

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To search for possible anti-tumor-promoters, fourteen flavones obtained from the root of *Scutellaria baicalensis* were examined for their inhibitory effects on the Epstein-Barr virus early antigen (EBV-EA) activation by a short-term *in vitro* assay. Among these flavones, 5,7,2′-trihydroxy- and 5,7,2′,3′-tetrahydroxyflavone showed remarkable inhibitory effects on the EBV-EA activation, and the effect of the latter on Raji cell cycle was also examined by flow cytometer. These two flavones exhibited remarkable inhibitory effects on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis test.

Keywords *Scutellaria baicalensis*; Labiatae; flavone; Epstein-Barr virus; Raji cell; flow cytometry; cell cycle; two stage carcinogenesis; anti-tumor-promoter

We previously[2,3] reported the inhibitory effects of eighty flavonoids (thirteen flavones, twenty-five flavanones, fourteen flavonols, one flavanonol, sixteen chalcones, nine isoflavones and two catechins) on the Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as a primary screening test for anti-tumor-promoting agents. As a result of our continuing search among medicinal plants for novel, naturally occurring, potential anti-tumor-promoters, the acetone extract of *Scutellaria baicalensis* was found to show significant inhibitory effects on EBV-EA activation. From the fraction eluted with ethyl acetate from the original acetone extract, fourteen flavones were isolated and their structures particularly related to 2′-oxygenative flavones reported.[4,5] In this paper, we report the results of the assay on inhibitory effects of these flavones on EBV-EA activation, the effect of **6** to the Raji cell cycle using a flow cytometer[6,7] and the inhibitory effects of **4** and **6** on mouse skin tumor promotion in an *in vivo* two stage carcinogenesis test.

TABLE I. Relative Ratio of EBV-EA Activation with Respect to Positive Control (100%) in Presence of Extracts and Fractions of *Scutellaria baicalensis*

Sample	Concentration[a]		
	100	10	1
n-Hexane ext.	0.0[b] (60)[c]	97.0 (>80)	100.0 (>80)
Acetone ext.	0.0 (20)	43.0 (>80)	100.0 (>80)
MeOH ext.	0.0 (>80)	77.0 (>80)	100.0 (>80)
n-Hexane fr.[e]	38.0 (>80)	97.0 (>80)	100.0 (>80)
$CHCl_3$ fr.[e]	64.0 (>80)	100.0 (>80)	100.0 (>80)
EtOAc fr.[e]	—[d] (0)	0.0 (>80)	67.0 (>80)
Acetone fr.[e]	0.0 (>80)	57.0 (>80)	100.0 (>80)
MeOH fr.[e]	0.0 (70)	34.0 (>80)	100.0 (>80)
100% H_2O fr.[f]	68.0 (70)	84.0 (>80)	100.0 (>80)
20% MeOHaq. fr.[f]	26.0 (>80)	100.0 (>80)	100.0 (>80)
40% MeOHaq. fr.[f]	0.0 (70)	48.0 (>80)	90.0 (>80)
60% MeOHaq. fr.[f]	0.0 (60)	100.0 (>80)	100.0 (>80)
100% MeOH fr.[f]	0.0 (50)	90.0 (>80)	100.0 (>80)

a) μg/ml (TPA: 20 ng = 32 pmol/ml). *b*) Values represent relative percentages to the positive control value. *c*) Values in parentheses are viability percentages of Raji cells. *d*) Not detected. *e*) Fraction of silica gel chromatography. *f*) Fraction of column chromatography on Diaion HP-20.

Results and Discussion

As shown in Table I, a fraction eluted with ethyl acetate from the original acetone extract of *S. baicalensis* exhibited significant inhibitory effects on EBV-EA activation even at a low dose (100% and 33% inhibition of the activation even at 10 μg/ml and 1 μg/ml, respectively). Other extracts and fractions exhibited less inhibitory effects than EtOAc fraction. An *in vitro* primary screening test of flavones (**1**—**14**) isolated from this EtOAc fraction was shown in Table II. The flavones (**3**, **4**, **6**, **8**, **10** and **13**) exhibited remarkable inhibitory effects on EBV-EA activation (100% inhibition at 1×10^3 mol ratio and more than 60% inhibition at 5×10^2 mol ratio of inhibitor/TPA) and preserved the high viability of Raji cells. Especially, 5,7,2′,3′-tetrahydroxyflavone (**6**) exhibited the most significant inhibitory activity among these flavones (100% inhibition at 5×10^2 mol ratio of inhibitor/TPA). In our experiments, the inhibitory activity of **6** was similar to those of retinoic and glycyrrhetinic acids which are known as strong anti-tumor-promoters.[8,9]

The effect of **6** on the cell cycle of Raji cells was then

TABLE II. Relative Ratio of EBV-EA Activation with Respect to Positive Control (100%) in Presence of Flavonoids

Sample	Concentration[a]			
	1×10^3	5×10^2	1×10^2	1×10
1	0.0[b] (>80)[c]	44.8 (>80)	90.3 (>80)	100.0 (>80)
2	0.0 (>80)	48.3 (>80)	89.4 (>80)	100.0 (>80)
3	0.0 (>80)	35.0 (>80)	65.9 (>80)	100.0 (>80)
4	0.0 (60)	36.3 (>80)	58.4 (>80)	100.0 (>80)
5	19.5 (>80)	95.5 (>80)	100.0 (>80)	100.0 (>80)
6	0.0 (>80)	0.0 (>80)	78.1 (>80)	90.6 (>80)
7	24.5 (>80)	62.5 (>80)	80.4 (>80)	100.0 (>80)
8	0.0 (>80)	27.4 (>80)	47.3 (>80)	81.2 (>80)
9	44.1 (>80)	61.5 (>80)	70.1 (>80)	100.0 (>80)
10	13.4 (>80)	37.9 (>80)	54.7 (>80)	88.8 (>80)
11	14.8 (>80)	32.9 (>80)	64.8 (>80)	100.0 (>80)
12	48.4 (>80)	84.3 (>80)	100.0 (>80)	100.0 (>80)
13	0.0 (>80)	37.1 (>80)	60.5 (>80)	100.0 (>80)
14	37.6 (>80)	50.5 (>80)	63.5 (>80)	100.0 (>80)

a) mol ratio/TPA (20 ng = 32 pmol/ml). *b*) Values represent relative percentages to the control value (100%). *c*) Values in parentheses are viability percentages of Raji cells.

TABLE III. Flow Cytometric Analysis of Raji Cell Cycle Treated with Compound **6**[a]

Phase	Positive[b] control	Medium[c] only	Treated with comp. **6**[d]		
			32 nmol	16 nmol	3.2 nmol
G_1	53.7	61.8	55.0	53.5	52.0
S	20.7	27.7	34.1	33.6	31.1
G_2+M	25.6	10.5	10.9	12.9	16.9
Total	100.0	100.0	100.0	100.0	100.0

a) Percentages of Raji cells in each phase. *b*) Treated with TPA (32 pmol) and *n*-butyric acid. *c*) Raji cells cultivated in RPMI-1640 medium containing 10% festal calf serum. *d*) Treated with TPA (32 pmol), *n*-butyric acid and compound **6** (32, 16 and 3.2 nmol).

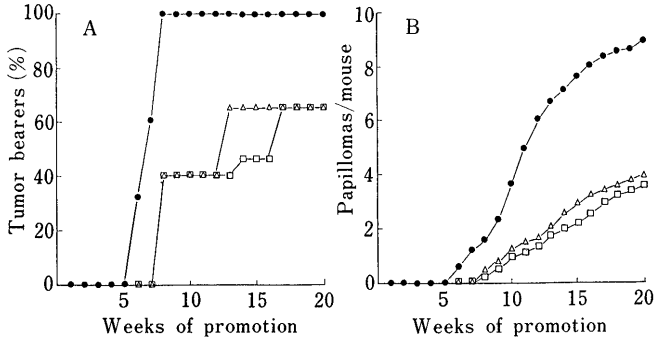
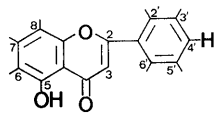


Fig. 1. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of Compounds **4** and **6** (85 nmol)

All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA given twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas. B. average number of papillomas per mouse. ●, control TPA alone; △, TPA + 85 nmol of **4**; □, TPA + 85 nmol of **6**.



	6	7	8	2′	3′	5′	6′
1	OH	OH	H	H	H	H	H
2	OMe	OH	H	H	H	H	H
3	H	OH	OMe	H	H	H	H
4	H	OH	H	OH	H	H	H
5	H	OH	H	OH	H	H	OH
6	H	OH	H	OH	OH	H	H
7	H	OMe	OMe	OH	H	H	H
8	OMe	OH	H	OH	H	H	H
9	H	OMe	OMe	OH	H	OH	H
10	H	OMe	OMe	OH	H	H	OH
11	H	OH	OMe	OH	H	H	OMe
12	OMe	OMe	OMe	OH	H	H	H
13	H	OMe	OMe	OH	H	H	OMe
14	OMe	OMe	OMe	OH	H	H	OMe

Chart 1. Structures of Flavonoids (**1**—**14**) Obtained from Scutellaria Plants

examined by flow cytometry. As shown in Table III, the promoter TPA increased the percentage of the G_2 and M phase of Raji cells and decreased the percentages of both the G_1 and S phase in comparison with negative control. When treated with compound **6**, the percentage of the S phase was on the increase and the percentage of G_2 and M phase was on the decrease as compared with the positive control. From these facts, it was deduced that compound **6** accumulated in Raji cells in the S phase, and that consequently, the percentage of the G_2 and M phase was restored to normal value.

On the basis of the results of *in vitro* assays (inhibitory effects on EBV-EA activation and effect on cell cycle), the effects of flavones (**4** and **6**) on two-stage carcinogenesis *in vivo* were investigated. The activities, evaluated by both the rate (%) of papilloma-bearing mice and average number of papillomas per mouse, were compared with those of a positive control. As shown in Fig. 1, both compounds **4** and **6**, when applied continuously before each TPA-treatment, delayed the formation of papillomas in mouse skin and reduced the rate of papilloma-bearing mice (about 30% reduction even at 20 weeks) as compared with the control experiment with TPA alone. These two compounds also reduced the number of papillomas per mouse (about 60% reduction even at 20 weeks). These results strongly suggested that 5,7,2′-trihydroxyflavone (**4**) and 5,7,2′,3′-tetrahydroxyflavone (**6**) might be valuable as anti-tumor-promotors in carcinogenesis.

Experimental

Fractionation and Isolation of Flavones (1—14) An acetone extract of the roots of *S. baicalensis* was subjected to column chromatographies on silica gel (eluent: C_6H_{14}, $CHCl_3$, EtOAc, acetone and MeOH, successively) and on Diaion HP-20 (eluent: water and mixtures of water increased in a portion of MeOH), separately. Each fraction with high activity was further purified with silica gel chromatography (eluent: $CHCl_3$–MeOH) to obtain flavones (**1**—**14**).[4] Two flavones (**4** and **6**) were synthesized.[5]

Biological Activities *in Vitro* The inhibition of EBV-EA activation was assayed using the EBV genome-carrying lymphoblastoid cell (Raji), which was cultivated in RMPI 1640 medium. The indicator cells were incubated at 37 °C for 48 h in 1 ml of the medium containing *n*-butyric acid (4 mM, co-inducer), TPA (20 ng/ml, 32 pM) and a known amount of test compound in dimethylsulfoxide (DMSO). Smears were made from the cell suspension. The activated cells were stained by high titer EBV positive sera from nasopharyngeal carcinoma (NPC) patients and detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted and the experiments were repeated twice. The average EA induction was compared to that of positive control experiments with *n*-butyric acid (4 mM) and TPA (32 pM), in which EA induction was ordinarily around 30%.

Flow Cytometric Analysis Cellular deoxyribonucleic acid (DNA) content of Raji cells was measured by flow cytometry. Fluorescence spectra were obtained and accomplished on a commercially available FACScan (Becton Dikinson). The cultured cells (1×10^6/ml) in plastic tubes were stained with propidium iodide by a rapid staining technique.[10] The nonionic detergent Triton × 100 (Nacalai tesque Co., Ltd.) 0.1% was added to the tubes for the purpose of lysis of the cell membrane. Treated Raji cells were filtered through a 37 m-pore nylon filter before staining. Treatment of ribonuclease (RNase, Sigma) in phosphate-buffered saline (PBS) (final 0.1%) decreased the fluorescence intensities of ribonucleic acid (RNA). Finally, we used propidium iodide (final: 50 μg/ml) for viable DNA staining. The flow cytometric analysis was carried out with a FACScan cell fit DNA system and the cell cycle pattern was analyzed by its program.

Two-Stage Carcinogenesis Test *in Vivo* Female ICR mice (7 weeks old) were obtained from Shizuoka Laboratory Animal Center, Shizuoka, Japan. Each group was composed of 15 mice housed 5 per cage and given H_2O *ad libitum*. The back of each mouse was shaved with surgical clippers. The mice were initiated with dimethylbenz[*a*]anthracene (DMBA, 100 μg, 390 nM) in Me_2CO. One week after initiation, they were promoted twice a week by application of TPA (1 μg, 1.7 nM) in Me_2CO. One hour before each TPA-treatment, the mice were treated with sample (85 nM or 50 μg, respectively) in Me_2CO. The incidence of papillomas was observed weekly for 20 weeks.

References and Notes

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