Anti-tumor Promoting Activities of Isoflavonoids from *Wistaria brachybotrys*¹⁾

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Eight minor isoflavonoids (3–10) isolated from the knot of *Wistaria brachybotrys* were tested for their inhibitory effects on Epstein-Barr virus (EBV) activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in Raji cells as a primary screening test for anti-tumor-promoters (cancer chemopreventive agents), and all the tested compounds showed inhibitory activity. Of these compounds, pendulone (3) was further examined on the cell cycle of Raji cells, and indicated strong inhibition on the effect of the cell cycle induced by TPA. In addition, the compound showed potent anti-tumor-promoting activity for an *in vivo* two-stage carcinogenesis test of mouse skin using 7,12-dimethylbenz[*a*]-anthracene and TPA. Consequently, it suggests that 3 could be a valuable chemopreventive agent in chemical carcinogenesis.

Key words pendulone; *Wistaria brachybotrys*; anti-tumor-promoter; Epstein-Barr virus; cell cycle; two-stage carcinogenesis

The knots of *Wistaria brachybotrys* SIEB. et ZUCC. (Leguminosae) have been used as a folk medicine in Japan for the treatment of gastric cancer.³⁾ In previous papers, we reported the isolation of nine triterpenoid saponins, including six new compounds from the knots.^{4a,b)} As a part of our continuing search for anti-tumor-promoters (cancer chemopreventive agents) from medicinal plants, primary screening tests for anti-tumor-promoters with the methanol extract,⁵⁾ saponins,^{4b,c)} and isoflavones⁶⁾ of the knot and their derivatives have been carried out. Of these compounds, afromosin (1)⁶⁾ and soyasaponin I (2)^{4b)} exhibited particularly potent inhibitory effects on Epstein-Barr virus-Early Antigen (EBV-EA) activation induced by a tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in Raji cells as a primary screening test for an anti-tumor-promoter. It is also demonstrated that 1 and 2 showed potent inhibitory effects on mouse

skin and/or pulmonary tumor promotion in two-stage carcinogenesis tests,⁷⁾ and the combination of 1 with 2 markedly enhanced the inhibitory effect *in vivo*.⁷⁾ These findings strongly suggest that 1 and 2 might be valuable anti-tumor-promoters in chemical carcinogenesis.

In this paper, we report the results of a primary screening test for the inhibitory effects of eight minor isoflavonoids (3–10) newly isolated from the knots of *Wistaria brachybotrys* on EBV-EA activation. The results of the cell cycle analysis on Raji cells and an *in vivo* two-stage carcinogenesis test on mouse skin tumor promotion with pendulone (3), which was potentially active on the inhibition of EBV-EA activation, are also reported.

MATERIALS AND METHODS

Test Compounds The compounds (3–10), as shown

Chart 1

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in Chart 1, were isolated from the knot of *Wistaria brachybotrys* SIEB. et ZUCC., purchased from Uchida Wakanyaku Co., Ltd.

All the compounds, pendulone (**3**) [mp 149–150 °C, $[\alpha]_D^{20} -42^\circ$ ($c=2.0$, MeOH)],^{8a,b)} isomucronulatol (**4**) [mp 144–145 °C, $[\alpha]_D^{20} -12^\circ$ ($c=1.0$, MeOH)],^{8c)} vestitol (**5**) [mp 156–157 °C, $[\alpha]_D^{20} -16^\circ$ ($c=1.0$, MeOH)],^{8a,d)} sativan (**6**) [mp 129–130 °C, $[\alpha]_D^{20} -13^\circ$ ($c=1.4$, MeOH)],^{8e)} medicarpin (**7**) [mp 132–133 °C, $[\alpha]_D^{23} -208^\circ$ ($c=0.9$, MeOH)],^{8f)} biochamin A (**8**) (mp 211–212 °C),^{8a,g)} irisolidon (**9**) (mp 191–192 °C),^{8a,h)} and 8-*O*-methylretusin (**10**) (mp 227–229 °C),^{8a,i)} were identified by melting point measurements and spectral data (IR, NMR, and MS).^{8j)}

Chemicals The cell culture reagents, *n*-butyric acid and other reagents, were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA, 7,12-dimethylbenz [*a*]anthracene (DMBA), and ribonuclease (RNase) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC), used for an immunofluorescence test, was a gift from Prof. H. Hattori, Department of Otorhinolaryngology, Kobe University.

Cells The EBV genome carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) under the conditions described previously.⁹⁾ Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.

Animals Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC, Inc. (Hama-matsu, Japan), and the animals were housed, five per polycarbonate cage, in a temperature-controlled room at $24 \pm 2^\circ\text{C}$ and given food and water *ad libitum*.

In Vitro EBV-EA Activation Experiments The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer), the EBV genome-carrying human lymphoblastoid cells which were cultivated in 10% fetal bovine serum (FBS) RPMI 1640 medium. The indicator cells (Raji, $1 \times 10^6/\text{ml}$) were incubated at 37 °C for 48 h in 1 ml of medium containing *n*-butyric acid (4 mmol, inducer), TPA [32 pmol: 2 μl of 20 ng/ml in dimethylsulfoxide (DMSO)], and various amounts of the test compounds dissolved in 5 μl of DMSO. Smears were made from the cell suspension. The EBV-EA inducing cells were stained with high titer EBV-EA positive serum from NPC patients and detected by an indirect immunofluorescence technique.¹⁰⁾ In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) among them was recorded. Triplicated assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was expressed by making a comparison with that of the positive control experiment (100%) which was carried out with *n*-butyric acid (4 mmol) plus TPA (32 pmol). In the experiments, the EBV-EA induction was ordinarily at around 35%, and these values were taken as the positive control (100%). Four millimolar *n*-butyric acid alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the trypan-blue staining method.

Cell Cycle Analysis Flow Cytometry The cellular deoxyribonucleic acid (DNA) content of Raji cells was

measured by flow cytometry. The samples were analyzed on a FAC Star Plus flow cytometer (Becton and Dickinson, Sunnyvale, CA, U.S.A.). The cells ($1 \times 10^6/\text{ml}$) in plastic tubes, cultured using the same method as in the EBV-EA inhibitory assay, were stained with propidium iodide by a rapid staining technique.¹¹⁾ The nonionic detergent Triton $\times 100$ (0.1%) was added to the tubes for the purpose of lysis of the cell membrane. Treated Raji cells were filtered through a 37 μ -pore nylon filter (Kyoshin Riko Co., Ltd., Tokyo, Japan) before staining. RNase in phosphate-buffered saline (PBS) (in final concentration, 0.1%) was treated for the reduction of the fluorescence intensities of RNA. Finally, propidium iodide (final: 50 $\mu\text{g}/\text{ml}$) was used for viable DNA staining. The flow cytometric analysis was carried out with an FAC Scan cell fit DNA system, and the cell cycle pattern was analyzed by its program.

In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas The animals were divided into three experimental groups, 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with DMBA (100 μg , 390 nmol) in acetone (0.1 ml). One week after the initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg , 1.7 nmol) in acetone (0.1 ml) on the skin. Group I received this TPA treatment alone, and groups II and III received a topical application of each test compound (85 nmol) and glycyrrhetic acid (85 nmol) in acetone (0.1 ml) 1 h before each TPA treatment, respectively. The incidence and numbers of papillomas were detected weekly for 20 weeks, as described previously.¹²⁾

RESULTS AND DISCUSSION

The primary screening test of **3–10** was carried out utilizing a short-term *in vitro* synergistic assay on EBV-EA activation. Their inhibitory effects on the EBV-EA activation induced by TPA and the viability of Raji cells are shown in Table 1.

In this assay, all compounds tested showed inhibito-

Table 1. Relative Ratio^{a,b)} of EBV-EA Activation with Respect to Positive Control (100%) in Presence of the Isoflavonoids (**3–10**) from the Knot of *Wistaria brachybotrys*

Samples	Concentration ^{c)}			
	1000	500	100	10
3	0.0 (>80)	0.0 (>80)	14.9 (>80)	93.7 (>80)
4	0.0 (70)	0.0 (>80)	12.7 (>80)	83.8 (>80)
5	0.0 (70)	0.0 (>80)	64.0 (>80)	100.0 (>80)
6	0.0 (70)	0.0 (>80)	88.2 (>80)	100.0 (>80)
7	0.0 (70)	0.0 (>80)	89.5 (>80)	100.0 (>80)
8	0.0 (60)	15.4 (>80)	65.1 (>80)	87.7 (>80)
9	0.0 (60)	17.8 (>80)	68.6 (>80)	100.0 (>80)
10	0.0 (60)	19.5 (>80)	87.4 (>80)	100.0 (>80)
Glycyrrhetic acid ¹⁴⁾	15.6 (70)	54.3 (>80)	100.0 (>80)	100.0 (>80)

a) Values represent relative percentages to the positive control value (100%).

b) Values in parentheses are viability percentages of Raji cells. c) Mol ratio/TPA (20 ng = 32 pmol/ml). The values of all compounds at 500 mol ratio/TPA and the values of **3** and **4** at 100 mol ratio/TPA were significantly different from the control value ($p < 0.05$) without cytotoxicity.

ry effects on EBV-EA activation without cytotoxicity on Raji cells.¹³ As shown in Table 1, all these compounds exhibited remarkable inhibitory effects (**3**–**7**: 100% inhibition of activation, and **8**–**10**: more than 80% inhibition of activation at 500 mol ratio/TPA). Of these compounds, an isoflavanquinone, **3** and an isoflavan, **4** showed the most potent inhibitory activity; more than 85% inhibition of activation was observed even at 100 mol ratio/TPA, and the high viability of the Raji cells was preserved, even at the highest concentration (1×10^3 mol ratio/TPA).¹³ Their inhibitory activities were much higher than those of glycyrrhetic acid, which is known to be a potent anti-tumor-promoter.¹⁴ Since the inhibitory effect on EBV-EA activation correlates well with the anti-tumor-promoting activity *in vivo*,¹⁵ this suggests that these isoflavanoids (**3** and **4**) may be anti-tumor-promoters.

The effects of **3** on the cell cycle of Raji cells treated with TPA were examined by flow cytometry. As shown in Table 2, the promoter TPA increased the percentage of the G₂ plus M phase of Raji cells and decreased the percentages of both G₁ and S phases compared with those of the negative control cultivated without TPA and *n*-butyric acid. By treatment with **3**, the percentage of S

phase was increased but the percentage of G₂ plus M phase was decreased at any concentration of **3** compared with the positive control treated with TPA and *n*-butyric acid. Accordingly, it is deduced that **3** may accumulate in Raji cells in the S phase, dependent on the concentration of **3**, which may result in restoration of the percentage of the G₂ plus M phase to a normal value. Therefore, it is concluded that **3** strongly inhibits one of the biological activities of TPA by influencing the cell cycle.

The inhibitory activity of **4** was a little higher than that of **3**, but the yield of **4** from the knot was deficient for the *in vivo* biological assay. On the basis of the results of the *in vitro* assays described above, the inhibitory effect of **3** on the two-stage carcinogenesis test of mouse skin using DMBA as an initiator and TPA as a promoter was investigated. The incidences (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Fig. 1A and B, respectively.

The incidence of papillomas in group I was highly significant at 100% of the mice at 9 weeks of promotion. Further, more than 3 and 9 papillomas were formed per mouse at 10 and 20 weeks of promotion, respectively. In group II, the formation of papillomas in mouse skin was delayed and the mean numbers of papillomas per mouse were reduced; only about 33 and 80% of mice bore papillomas at 10 and 20 weeks of promotion, respectively, and 1.1 and 5.2 papillomas per mouse were detected at 10 and 20 weeks of promotion, respectively. Consequently, **3** exhibited about 42% inhibition even at 20 weeks of promotion, as shown in Fig. 1B. On the other hand, in group III, 46 and 93% of mice bore papillomas at 10 and 20 weeks of promotion, respectively, and 2 and 5.6 papillomas per mouse were detected at 10 and 20 weeks of promotion, respectively, indicating that the inhibitory activity of **3** for the two-stage carcinogenesis is higher than that of glycyrrhetic acid.

The results of the present study suggest that pendulone isolated from *W. brachybotrys* could be a valuable anti-tumor-promoter in chemical carcinogenesis.

Table 2. Flow Cytometric Analysis of Raji Cell Cycle Treated with **3**

Treatment	% of Raji cells				
	Phase	G ₁	S	G ₂ + M	Total
None ^{a)}		61.7	27.9	10.4	100.0
TPA (Positive control) ^{b)}		53.7	18.7	27.6	100.0
3 ^{c)}	32.0 nmol ^{d)}	55.0	35.1	9.9	100.0
	16.0 nmol	53.5	33.1	13.5	100.0
	3.2 nmol	53.9	24.5	21.6	100.0

a) Raji cells cultivated in RPMI-1640 medium (1 ml) containing 10% fetal calf serum. b) Treated with TPA (32 pmol) and *n*-butyric acid. c) Treated with TPA (32 pmol), *n*-butyric acid, and **3**. d) 32, 16, and 3.2 nmol are equal to 1000, 500, and 100 mol ratio/TPA, respectively. The values of **3** on S and G₂ + M phase at 32.0 and 16.0 nmol were significantly different from the positive control values ($p < 0.05$).

Fig. 1. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of **3** and Glycyrrhetic Acid

All mice were carcinogenically 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 (group I); ●, TPA + 85 nmol of **3** (group II); ○, TPA + 85 nmol of glycyrrhetic acid (group III). At 20 weeks of promotion, group II was different from group I ($p < 0.05$) on papillomas per mouse.

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