Cancer Chemopreventive Agents (Antitumor-promoters) from *Ajuga decumbens*

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Sixteen compounds (**1**−**16**) isolated from the flowering whole plant of *Ajuga decumbens* have been tested for their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) induction by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as a primary screening test for antitumor-promoters (potential cancer chemopreventive agents). Five compounds (**6**, **9**, and **12**−**14**) showed strong inhibitory effects on EBV-EA induction. Of these active compounds, two major constituents of this plant, cyasterone (**6**) and 8-acetylharpagide (**13**), showed potent antitumor-promoting activities on a mouse-skin in vivo two-stage carcinogenesis procedure, using 7,12-dimethylbenz[*a*]anthracene as initiator and TPA as promoter. Further, compound **13** also exhibited potent chemopreventive activity in a mouse pulmonary tumor model.

Cancer currently remains a very serious disease and is one of the major causes of death in the world, despite the many advances that have been made in cancer chemotherapy and other approaches to treatment. However, the ideal effective anticancer agent free from any side-effects has not yet been found. Therefore, cancer chemoprevention has become increasingly important in recent years. In our search for novel cancer chemopreventive agents (antitumor-promoters) from medicinal plants, we have carried out a primary screen followed by a two-stage carcinogenesis assay on many kinds of natural products (diterpenes,[1] triterpenes,[2−4] flavonoids,[4,5] euglobals,[6] and kampo prescription ingredients[7,8]) using their inhibitory effects on mouse skin tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) as the initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as the promoter.

The flowering whole plants of *Ajuga decumbens* Thunb. (Labiatae) have been used as a folk medicine for antiinflammatory, antitussive, and expectorant effects in the People's Republic of China and Japan.[9,10] In a previous paper,[11] we reported the isolation and structure elucidation of several new glycosides from this plant. In the course of our continuing search for cancer chemopreventive agents, these compounds were tested for their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) induction by the tumor promoter, TPA, in Raji cells as a primary screening test. Furthermore, 16 other compounds (**1**−**16**) were also isolated from this plant, and the primary screening was carried out. Of these compounds, cyasterone (**6**), polypodine B (**9**), decumbesterone A (**12**), 8-acetylharpagide (**13**), and loliolide (**14**) showed strong inhibitory effects on EBV-EA induction. Moreover, the compounds available in the largest amounts (**6** and **13**) among these active constituents, exhibited potent inhibitory effects on mouse skin-tumor promotion in a two-stage carcinogenesis assay, and **13** also exhibited significant inhibitory effects on mouse pulmonary-tumor promotion in a two-stage carcinogenesis procedure using 4-nitroquinoline-*N*-oxide (4NQO) as the initiator and 8% glycerol as the promoter.

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Table 1. Percentage of Epstein−Barr Virus Early Antigen Induction in the Presence of Compounds **1**−**16** with Respect to a Positive Control

	concentration (mol ratio/TPA)[a]			
compound	1000	500	100	10
1	10.6 ± 0[b] (70)[c]	54.7 ± 1.2	79.2 ± 1.5	100.0 ± 0.2
2	0.0 ± 0 (60)	42.6 ± 1.1	73.5 ± 1.3	100.0 ±0
3	0.0 ± 0.1 (50)	55.8 ± 1.4	76.3 ± 1.6	100.0 ±0
4	18.4 ± 0.6 (70)	47.2 ± 2.0	71.5 ± 1.8	100.0 ±0.1
5	0.0 ± 0 (70)	51.9 ± 1.6	80.2 ± 0.9	100.0 ±0
6	0.0 ± 0 (70)	36.2 ± 0.4	75.2 ± 1.0	94.6 ± 0.4
7	20.9 ± 0.4 (60)	61.9 ± 1.8	84.5 ± 0.9	100.0 ± 0.6
8	17.5 ± 0.5 (60)	58.2 ± 2.2	81.0 ± 0.8	100.0 ± 0.1
9	0.0 ± 0.2 (70)	23.4 ± 1.1	65.9 ± 2.2	100.0 ± 0.4
10	0.0 ± 0 (70)	73.7 ± 1.4	90.1 ± 0.5	100.0 ±0
11	4.6 ± 2.1 (70)	49.1 ± 1.3	72.6 ± 2.0	95.8 ± 1.3
12	0.0 ± 0 (70)	24.7 ± 0.7	66.0 ± 1.7	100.0 ±0
13	0.0 ± 0.1 (60)	19.9 ± 0.4	62.6 ± 1.0	89.5 ± 0.6
14	5.1 ± 1.8 (60)	26.8 ± 2.6	59.6 ± 1.6	88.0 ± 1.7
15	31.5 ± 2.0 (50)	65.9 ± 1.7	83.7 ± 1.2	100.0 ± 0.3
16	0.0 ± 0.4 (60)	46.2 ± 1.5	73.9 ± 1.4	90.2 ± 0.9

[a] Mole ratio/TPA (32 pmol = 20 ng/mL), 1000 mol ratio = 32 nmol, 500 mol ratio = 16 nmol, 100 mol ratio = 3.2 nmol, and 10 mol ratio = 0.32 nmol. [b] Values represent percentages of EBV-EA induction to the positive control values (100%) (n = 3 and ± S.D.). [c] Values in parentheses represent viability percentages of Raji cells; unless otherwise stated, the viability percentages of Raji cells were more than 80%.

Results and Discussion

The MeOH extract of *A. decumbens* was extracted successively with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, and each extract was screened for inhibitory effects on EBV-EA induction. The MeOH, *n*-hexane, $CHCl_3$, and *n*-BuOH extracts showed remarkable inhibitory effects on EBV-EA induction,[12] and 16 compounds isolated from these extracts were tested for primary screening. Of these constituents, as shown in Table 1, compounds **6**, **9**, and **12**−**14** exhibited potent inhibitory effects (100% inhibition of induction at 1000 mol ratio/TPA, and more than 60% and about 30% inhibition even at 500 mol ratio/TPA and 100 mol ratio/TPA, respectively) on EBV-EA induction by TPA.[11,13] In our hands, the inhibitory effects of these compounds were found to be stronger than those of glycyrrhetic acid and retinoic acid, which are known to be strong antitumor-promoters.[14,15] Compound **13**, in particular, exhibited significant inhibitory effects (100% and more

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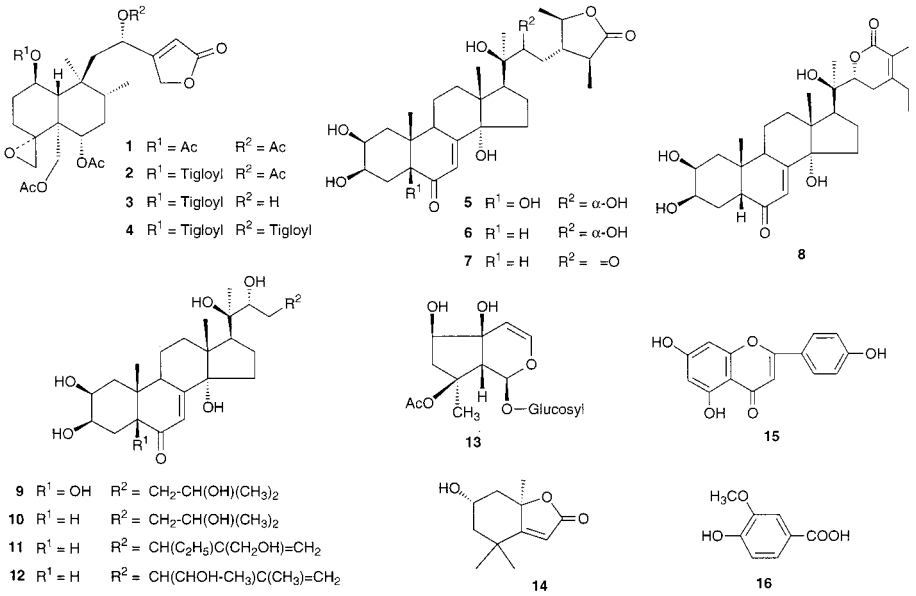

than 80% inhibition at 1000 mol ratio/TPA and 500 mol ratio/TPA, respectively) and preserved high viability of Raji cells. On the other hand, for seven compounds (**1**, **3**, **5**, **7**, **8**, **10**, and **15**), strong inhibitory effects on EBV-EA induction were not observed (more than 50% and 80% EBV-EA induction at 500 and 100 mol ratio/TPA, respectively). In our past work, inhibitory effects on EBV-EA induction have correlated well with antitumor-promoting activity in vivo.[1−8] Compounds **6** (0.02% of dried material) and **13** (0.17% of dried material) were the major constituents, while the other active compounds were minor constituents (less than 0.01% of dried material) of *A. decumbens*. Accordingly, we investigated the inhibitory effects of **6** and **13** in a two-stage carcinogenesis test on mouse skin using DMBA as initiator and TPA as promoter. The incidence (%) of papilloma-bearing mice and the average number of papillomas per mouse are presented and compared with a positive control and glycyrrhetic acid in Figures 1 and 2, repectively.

In the positive control experiment (initiated with 390 nmol of DMBA and promoted with TPA), 80% of mice bore papillomas after 8 weeks of promotion, and all mice bore papillomas after 10 weeks of promotion as shown in Figures 1A and 2A. Further, in the positive control experiment, more than 4 and 9 papillomas were formed per mouse after 10 and 20 weeks of promotion, respectively (Figures in 1B and 2B).

When **6** and **13** were applied before each TPA treatment, they remarkably delayed the formation of papillomas and reduced the number of papillomas per mouse as follows. In the group treated with **6**, only 43%, 60%, and 80% of mice bore papillomas after 8, 10, and 17 weeks of promotion, respectively. Also, fewer than 2.5 and 7 papillomas were formed per mouse after 10 and 20 weeks, in turn, of promotion. Further, in the group treated with **13**, only 10%, 20%, and 80% of mice bore papillomas at 8, 10, and 17 weeks of promotion, respectively, while fewer than 2 and 6 papillomas were formed per mouse after 10 and 20 weeks of promotion, respectively. Therefore, compound **13** exhibited about 35% inhibition, even at 20 weeks of promotion as shown in Figure 2B, and the inhibitory effects of **13** on the two-stage carcinogenesis of mouse skin tumors promoted by TPA were apparently more potent than those of glycyrrhetic acid.

Furthermore, the effects of **13** were assayed in a two-stage carcinogenesis model involving pulmonary tumor formation, in which 4NQO and 8% glycerol were used as an initiator and promoter, respectively. In the positive control (group IV), pulmonary tumors were formed in 80% of mice and 42 tumors were formed in total in 15 mice after 22 weeks of promotion, as shown in Table 2. On the other hand, both the total number of pulmonary tumors in 15 mice and the percentage of mice with tumors were markedly reduced in a concentration-dependence test for compound **13**. In group V, treated with 0.0025% of **13**, 53.3% of mice bore pulmonary tumors, and only 17 tumors were formed (1.1 tumors per mouse) after 22 weeks of promotion. Further, in group VI, treated with 0.005% of **13**, only 46.6% of mice bore pulmonary tumors, and only 14 tumors were formed (0.9 tumors per mouse) in total in 15 mice after 22 weeks of promotion.

These in vivo results suggest that 8-acetylharpagide (**13**) might be valuable as an antitumor-promoter and a chemopreventive agent in chemical carcinogenesis. In addition, on the basis of the high yields of **13** (0.17%) in the flowering whole plant of *A. decumbens*, this species may be valuable as a source of this potential cancer chemopreventive agent.

Experimental Section

Plant Material and Test Compounds. Four neoclerodane diterpenes (**1**−**4**), eight ecdysteroids (**5**−**12**), an iridoid glycoside (**13**), and three miscellaneous substances (**14**−**16**) were isolated and structurally characterized from the flowering whole plant of *Ajuga decumbens*.[11,13,16−23] A voucher specimen of the plant and the details of extraction were reported in our previous paper.[11]

Chemicals. The cell culture reagents and *n*-butyric acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenz[*a*]anthracene (DMBA) were obtained from Sigma Chemical Co. (St. Louis, MO), and 4-nitroquinoline-*N*-oxide (4NQO) and glycerol were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Animals. Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC Inc. (Hamamatsu, Japan), and the animals were housed, five per polycarbonate cage, in a temperature-controlled room at 24 ±2 °C and given food and water ad libitum.

In Vitro EBV-EA Induction Effect. The EBV genome-carrying lymphoblastoid cells, Raji cells, derived from Barkitt's lymphoma, were cultivated in RPMI-1640 medium. The Raji cells were incubated for 48 h at 37 °C in a medium containing *n*-butyric acid (4 mmol), TPA (32 pmol), and various amounts

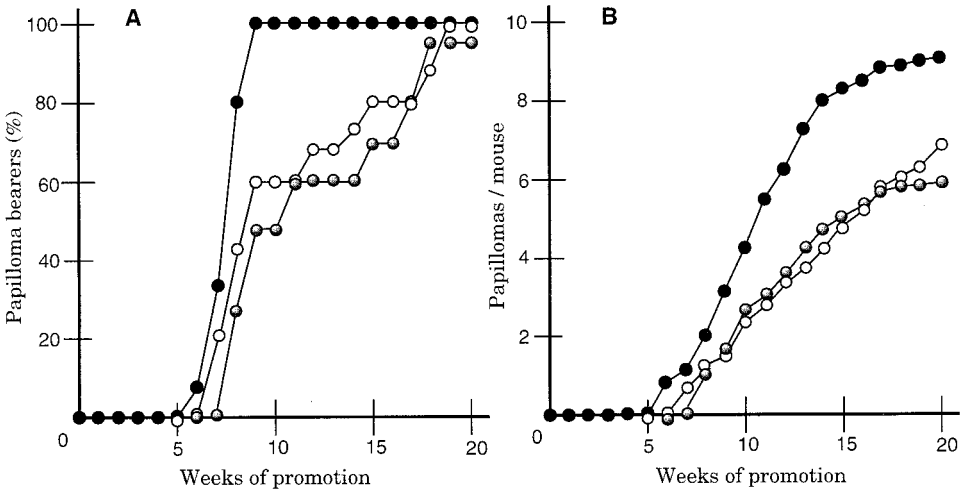

Figure 1. Inhibition of TPA-induced tumor promotion by multiple applications of cyasterone (**6**) and glycyrrhetic acid. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. **A**: percentage of mice bearing papillomas, **B**: average number of papillomas per mouse. ●, control TPA alone; ◒, TPA + 85 nmol of glycyrrhetic acid; ○, TPA + 85 nmol of **6**. At 20 weeks of promotion, groups treated with **6** and glycyrrhetic acid were different from the control group ($p < 0.05$) in terms of the number of papillomas per mouse.

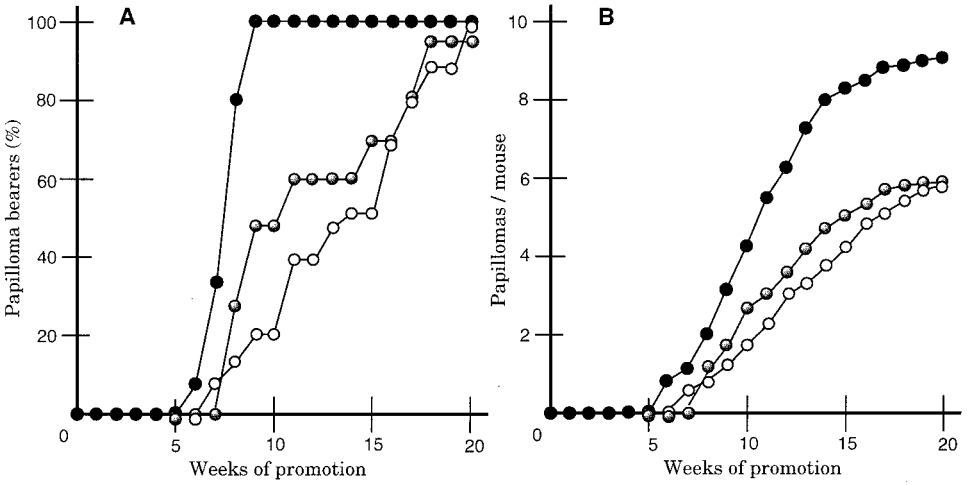

Figure 2. Inhibition of TPA-induced tumor promotion by multiple application of 8-acetylharpagide (**13**) and glycyrrhetic acid. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. **A**: percentage of mice bearing papillomas, **B**: average number of papillomas per mouse. ●, control TPA alone; ◒, TPA + 85 nmol of glycyrrhetic acid; ○, TPA + 85 nmol of **13**. At 20 weeks of promotion, groups treated with **13** and glycyrrhetic acid were different from the control group ($p < 0.05$) in terms of the number of papillomas per mouse.

of test compounds. Smears were made from the cell suspension, and the EBV-EA-inducing cells were stained by means of an indirect immunofluorescence technique. The details of the in vitro assay on EBV-EA induction have been reported previously.[6,7,11]

In Vivo Two-Stage Carcinogenesis Method for Mouse Skin Papillomas Promoted by TPA. The animals were divided into three experimental groups, at 15 mice each. The backs of the mice were shaved with surgical clippers, and they were treated topically with DMBA (100 μg, 390 nmol) in Me_2CO (0.1 mL) as the initiation treatment. One week after initiation, papilloma formation was promoted twice a week by the application to the skin of TPA (1 μg, 1.7 nmol) in Me_2CO (0.1 mL). Group I received the TPA treatment alone, and groups II, III, and IV received a topical application of cyasterone (**6**, 85 nmol), 8-acetylharpagide (**13**, 85 nmol), and glycyrrhetic acid (85 nmol) in Me_2CO (0.1 mL), respectively, 1 h before the TPA treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

In Vivo Two-Stage Carcinogenesis Method for Mouse Pulmonary Tumors Promoted by Glycerol.[24] All mice were given commercial rodent pellets and either tap water or water containing glycerol (8 %) ad libitum. Ninety mice were divided into six groups of 15 animals each. Each experimental group received initiation/promotion treatments with the following agents: (I) drinking water alone, $n = 15$; (II) 8% glycerol solution alone, $n = 15$; (III) 4NQO/water, $n = 15$; (IV) 4NQO/8% glycerol solution, $n = 15$; (V) 4NQO/8% glycerol and

Table 2. Incidence of Pulmonary Tumors in Mice Treated with 8-Acetylharpagide (**13**)[a]

group	treatment	total number of tumors	tumors per mouse	mice with tumors (%)
I	water	0.0	0.0	0.0
II	8% glycerol	0.0	0.0	0.0
III	4NQO + water	4.0[b]	0.26[b]	13.3[b]
IV	4NQO + 8% glycerol	42.0	2.8	86.7
V	4NQO + 8% glycerol + 0.0025% of **13**	17.0[b]	1.1[b]	53.3
VI	4NQO + 8% glycerol + 0.005% of **13**	14.0[b]	0.9[b]	46.6[b]

[a] Initiator: 4-nitroquinoline-*N*-oxide (4NQO), 0.3 mg per mouse, subcutaneous injection; promoter: 8% glycerol in drinking water. The mean intake of drinking water was 7.0, 7.1, 7.0, 7.1, 7.1, and 7.1 mL/mouse/day in group I to VI, respectively, and no statistically significant difference was observed between each group. The body weight of the animals was not affected by treatment with **13** in the experiments. At the end of this experiment, the average of body weight of mice was 47.0 ± 4.8, 50.1 ± 5.2, 48.3 ± 4.7, 50.2 ± 4.8, 49.8 ± 4.8, and 49.7 ± 4.9 g in group I to VI, respectively. [b] Statistically different from the control group (IV), $p < 0.05$, using Student's *t*-test.

compound **13** (2.5 mg/100 mL), $n = 15$; (VI) 4-NQO/8% glycerol and compound **13** (5 mg/100 mL), $n = 15$. The total dose of **13** was 1.27 and 2.54 mg/mouse/week in groups V and VI, respectively, and that of glycerol was 3.9 g/mouse/week in both groups.

Initiation. 4NQO was dissolved in olive oil containing cholesterol (20:1). A dose of 0.3 mg of 4NQO was given to each mouse by a single subcutaneous injection at the start (groups III, IV, and V). In the control mice, the same amount of the vehicle was administered at the start (groups I and II).

Promotion. Glycerol was dissolved in water (8%). Five weeks after initiation, the promoting treatment with glycerol solution (groups II and IV) or with the glycerol solution containing compound **13** (groups V and VI) was started by administering the solution to mice as drinking water ad libitum. The treatment was continued for 22 weeks. Other groups (I and III) were given only tap water ad libitum. The amount of solution consumed was checked twice a week.

Treatment of Animals. The animals were killed by cervical dislocation after a 22-week promotion period. Each pulmonary lobe was separated, and the number of induced tumors was counted under a dissecting microscope. The lungs were embedded in paraffin, sectioned, and stained with hematoxylin eosin by the conventional method, to allow the histological study of the pulmonary tissue.

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(12) The relative ratio of EBV-EA induction in the presence of extracts with respect to a positive control (100%) at 100 μg/mL, 10 μg/mL, and 1 μg/mL were followed. MeOH extract, 0, 54.1, and 88.6; *n*-hexane extract, 0, 27.5, and 87.3; $CHCl_3$ extract, 7.9, 54.5, and 97.1; EtOAc extract, 16.7, 86.5, and 100; *n*-BuOH extract, 0, 27.3, and 75.9, respectively. In these experiments, the viability percentages of Raji cells were more than 70% at every concentration.
(13) Chemical structures and results on EBV-EA inductions of other glycosides have been reported in a previous paper.[10]
(14) Percentages of EBV-EA induction in the presence of glycyrrhetic acid were 15.6, 54.3, 100, and 100% at 1×10^3, 5×10^2, 1×10^2, and $1 \times$ 10 mol ratio/TPA, respectively, and the viability percentage of Raji cells was more than 80% at each concentration.
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