Anti-tumor-Promoting Activities of Triterpenoids from Ferns. I

Takao KONOSHIMA,*^a Midori TAKASAKI,^a Harukuni TOKUDA,^b Kazuo MASUDA,^c
Yoko ARAI,^c Kenji SHIOJIMA,^c and Hiroyuki AGETA^c

Kyoto Pharmaceutical University,^a Misasagi, Yamashina-ku, Kyoto 604, Japan, Kyoto Prefectural University of Medicine,^b Kawaramachi-hirokoji, Kamigyo-ku, Kyoto 602, Japan, and Showa College of Pharmaceutical Sciences,^c Machida, Tokyo 194, Japan. Received January 29, 1996; accepted April 1, 1996

To search for possible anti-tumor-promoters (cancer chemopreventive agents), we carried out primary screening of 23 triterpenoid hydrocarbons (1—23) isolated from ferns using an *in vitro* synergistic assay system. Of these triterpenoids, hop-17(21)-ene (2), neohop-13(18)-ene (3), neohop-12-ene (4), taraxerane (17), multiflor-9(11)-ene (18), multiflor-8-ene (19), glutin-5(10)-ene (21) and taraxastane (23) exhibited remarkable inhibitory effects on Epstein-Barr virus (EBV) activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Further, compounds 2 and 3 exhibited remarkable anti-tumorpromoting effects on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis test using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter.

Key words anti-tumor-promoter; triterpenoid hydrocarbon; hop-17(21)-ene; neohop-13(18)-ene; fern; mouse skin tumor

The mechanism of chemical carcinogenesis has been explained by either a two-stage theory or a multi-stage theory,¹⁾ and the development of anti-tumor-promoters has been regarded as the most effective method for the chemoprevention of cancer.²⁾ As a continuation of our chemical and biological studies on potential anti-tumor-promoters (chemopreventive agents), we carried out primary screening of many natural products (euglobals,³⁾ flavonoids,⁴⁾ quinones,⁵⁾ triterpenoid saponins⁶⁾ and crude drugs⁷⁾ using their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). It has also been reported that many compounds which inhibit EBV-EA induction by tumor promoters have been shown to act as inhibitors of tumor promotion *in vivo*.⁸⁾ Furthermore, some natural products which exhibited anti-tumor-promoting activities on a two-stage carcinogenesis test of mouse skin tumor by the application have also exhibited inhibitory effects on two-stage carcinogenesis tests of mouse pulmonary and mouse hepatic tumors by oral administration.⁹⁾

In previous papers,¹⁰⁾ we reported the inhibitory effects of 50 cucurbitacins and their derivatives on EBV-EA activation and the inhibitory effects of several cucurbitacin derivatives on the two-stage carcinogenesis test. Further, Ageta and his colleagues have reported the isolation and structural elucidation of many pentacyclic triterpenoids from ferns.^{11–15)}

In this paper, we report the results of a primary screening test *in vitro* on the inhibitory effects of 23 triterpenoid hydrocarbons (1—23) belonging to hopane, migrated hopane, oleanane and migrated oleanane groups from *Adiantum*, *Dryopteris*, *Polypodium* and *Oleandra* genera on EBV-EA activation, as well as the results of a two-stage carcinogenesis test *in vivo* of hop-17(21)-ene (2) and neohop-13(18)-ene (3) on mouse skin tumor promotion.

MATERIALS AND METHODS

Cells EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured

in RPMI-1640 medium (Nissui) under the conditions described before.^{3–7)} Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.

Chemicals The tissue culture reagents, *n*-butyric acid and other reagents were purchased from Nacalai Tesque (Kyoto, Japan). TPA and 7,12-dimethylbenz[*a*]anthracene (DMBA) were obtained from Sigma Chemical Co. (U.S.A.). EBA-EA positive serum, from a patient with nasopharyngeal carcinoma used for an immunofluorescence test, was a gift from the Department of Otorhinolaryngology, Kobe University.

Animals Specific pathogen-free female ICR mice (6 weeks old) were obtained from Nippon SLC Co., Ltd. (Shizuoka, Japan), and housed in polycarbonate cages in a temperature-controlled room.

Triterpenoid Hydrocarbons Compounds 1—3, 5, 6, 9 and 14—23 were isolated from the rhizomes of *Polypodium nipponica*, *P. formosanum*, *P. vulgare*, *P. fauriei* and *P. virginianum*.^{11,14)} Compounds 7, 10 and 11 were isolated from the leaflets of *Dryopteris crassirhizoma*.¹³⁾ Compounds 4, 8 and 12 were isolated from the leaves of *Adiantum monochlamys*, and compound 13 was isolated from the rhizomes of *Oleandra wallichii*.^{12,15)}

In Vitro EBV-EA Activation Experiment 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 of the test compounds. Smears were made from the cell suspension, and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique.¹⁶⁾ The details of the *in vitro* assay on EBV-EA activation have been reported previously.¹⁰⁾ In each assay, at least 500 cells were counted and the number of stained cells (positive cells) among them was recorded. Triplicate assays were performed for each data point.

In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas The mice were carcinogenically initiated by the application of DMBA (100 µg, 390 nmol) in acetone (0.1 ml), and promoted twice a week by the application of TPA (1 µg, 1.7 nmol) in acetone (0.1 ml). The *in vivo* two-stage carcinogenesis test on mouse skin papillomas was carried out according to the reported method,¹⁰⁾ and

* To whom correspondence should be addressed.

Chart 1

the incidence of papillomas was observed weekly for 20 weeks.

RESULTS AND DISCUSSION

The primary screening test of pentacyclic triterpenoid hydrocarbons obtained from ferns was carried out utilizing a short-term *in vitro* assay on EBV-EA activation induced by a promoter, TPA. The inhibitory effects of these triterpenoids on the activation and the viability of indicator cells, Raji cells, are shown in Table 1.

All of these triterpenoid hydrocarbons preserved the high viability of Raji cells, and compounds 2–5, 7–9 and 16–23 exhibited remarkable inhibitory effects on EBV-EA activation at a higher concentration and, in our experiments, these inhibitory activities were stronger than those of glycyrrhetic acid, which is known to be a strong

anti-tumor-promoter.¹⁷⁾ Further, compounds 2–4, 17–19, 21 and 23 showed more than 75% and 35% inhibition of activation at 5×10^2 and 1×10^2 mol ratio/TPA, respectively (especially, compounds 2 and 21 exhibited more than 50% inhibition of activation, even at 1×10^2 mol ratio/TPA, and 2 exhibited the strongest inhibitory effects). On the other hand, compounds 1, 6–10, 13–15 and 22 showed less than 20% inhibition at 1×10^2 mol ratio/TPA. Of these triterpenes, as in the case of compounds 10–14, oxygenated functional groups reduced the inhibitory effects on EBV-EA activation. The results *in vitro* of these eight triterpenoid hydrocarbons (2–4, 17–19, 21 and 23) strongly suggested that these compounds might be valuable anti-tumor-promoters as well. Considering the results *in vitro* and the yields of these triterpenoids, the inhibitory effects of hop-17(21)-ene (2) and neohop-13(18)-ene (3) on two-stage carcinogenesis of mouse skin

papillomas, using DMBA as an initiator and TPA as a promoter, were investigated. The activities, evaluated by both the rate (%) of papilloma-bearing mice (as shown in Fig. 1A) and the average number of papillomas per mouse (Fig. 1B), were compared with those of the positive control group and those of the control group treated with glycyrrhetic acid.

In the positive control group, 60%, 80% and 100% of mice bore papillomas at 8, 9 and 10 weeks of promotion,

Table 1. Relative Ratio of EBV-EA Activation with Respect to Positive Control in Presence of Triterpenes (1—23)

Sample	Concentration (mol ratio/TPA)			
	1×10^3	5×10^2	1×10^2	1×10
1	0.0 ^a (70) ^b	62.8 (>80)	84.0 (>80)	100.0 (>80)
2	0.0 (70)	24.3 (>80)	49.2 (>80)	68.0 (>80)
3	0.0 (70)	14.9 (>80)	63.1 (>80)	100.0 (>80)
4	0.0 (70)	17.7 (>80)	63.1 (>80)	87.3 (>80)
5	0.0 (70)	29.8 (>80)	68.0 (>80)	100.0 (>80)
6	16.6 (70)	61.0 (>80)	87.4 (>80)	100.0 (>80)
7	0.0 (70)	19.5 (>80)	80.2 (>80)	100.0 (>80)
8	0.0 (70)	17.3 (>80)	82.8 (>80)	100.0 (>80)
9	0.0 (70)	0.0 (>80)	80.4 (>80)	100.0 (>80)
10	26.9 (70)	58.4 (>80)	93.1 (>80)	100.0 (>80)
11	15.6 (70)	34.7 (>80)	65.9 (>80)	100.0 (>80)
12	18.1 (70)	29.6 (>80)	58.5 (>80)	93.3 (>80)
13	25.4 (70)	47.8 (>80)	100.0 (>80)	100.0 (>80)
14	17.3 (70)	42.5 (>80)	86.3 (>80)	100.0 (>80)
15	21.8 (70)	83.9 (>80)	100.0 (>80)	100.0 (>80)
16	0.0 (70)	18.6 (>80)	67.3 (>80)	89.0 (>80)
17	0.0 (70)	15.8 (>80)	62.7 (>80)	82.6 (>80)
18	0.0 (70)	14.3 (>80)	59.0 (>80)	78.4 (>80)
19	0.0 (70)	14.0 (>80)	55.3 (>80)	75.9 (>80)
20	0.0 (70)	22.7 (>80)	65.7 (>80)	88.0 (>80)
21	0.0 (70)	25.9 (>80)	47.8 (>80)	89.1 (>80)
22	0.0 (70)	29.6 (>80)	83.2 (>80)	100.0 (>80)
23	0.0 (70)	17.5 (>80)	57.0 (>80)	79.2 (>80)

a) Values represent relative percentages to the positive control (100%). b) Values in parentheses are viability percentages of Raji cells.

respectively, and more than 9.5 papillomas were formed per mouse after 20 weeks of promotion, as shown in Fig. 1. When hop-17(21)-ene (2) and neohop-13(18)-ene (3) were applied before each TPA treatment, respectively, they delayed the formation of papillomas as follows. In the group treated with 2, only about 25% and 60% of mice bore papillomas at 10 weeks and 16 weeks of promotion, respectively, and 80% of the mice bore papillomas at 20 weeks of promotion. In the group treated with 3, only about 40% of mice bore papillomas at 10 weeks and 95% at 20 weeks of promotion. Therefore, the delay effect of 2 on papilloma formation was stronger than that of glycyrrhetic acid, and the delay effect of 3 was similar to that of glycyrrhetic acid. Also, compounds 2 and 3 reduced the number of papillomas per mouse as follows. In the case of 2, less than 1 and 4 papillomas were formed per mouse after 10 and 15 weeks of promotion, respectively, and only about 6 papillomas were formed, even at after 20 weeks of promotion. Further, in the case of 3, less than 2 and 5 papillomas were formed per mouse after 10 and 15 weeks of promotion, respectively, and about 6.7 papillomas were formed after 20 weeks of promotion. Compounds 2 and 3 exhibited about 40% and 35% inhibition, respectively, even after 20 weeks of promotion, as shown in Fig. 1. These results *in vivo* suggested that the inhibitory effects of hop-17(21)-ene (2) and neohop-13(18)-ene (3) on two-stage carcinogenesis were stronger than or similar to those of glycyrrhetic acid, therefore these compounds might be valuable as anti-tumor-promoters in chemical carcinogenesis.

The inhibitory effects of these compounds on the two-stage carcinogenesis of mouse pulmonary and mouse hepatic tumors by oral administration will be assayed. The inhibitory mechanisms of these triterpenoid hydrocarbons on tumor promotion and their structure-activity relationships are now being studied.

Fig. 1. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of Hop-17(21)-ene (2), Neohop-13(18)-ene (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 numbers of papillomas per mouse. ●, control TPA alone; ○, TPA + 85 nmol of hop-17(21)-ene (2); □, TPA + 85 nmol of neohop-13(18)-ene (3); ●, TPA + glycyrrhetic acid.

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