Anti-tumor-promoting Activity of Majonoside-R2 from Vietnamese Ginseng, Panax vietnamensis Ha et Grushv. (I.1)

Takao Konoshima,⁎ Midori Takasaki, Harukuni Tokuda, Hoyoku Nishino, Nguyen Minh Duc, Ryoji Kasai, and Kazuo Yamasaki

Kyoto Pharmaceutical University, Misaasagi, Yamashina-ku, Kyoto 607–8414, Japan, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyō-ku, Kyoto 602, Japan, Ho Chi Minh City University of Medicine and Pharmacy, 41 Dinh Tien Hoang, District 1, Ho Chi Minh City, Vietnam, and Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima 734–8551, Japan.

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Seven saponins (1—7) isolated from the rhizomes and roots of Panax vietnamensis were tested for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) 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). The oocititol-type saponin, majonoside-R2 (2), which is the major and characteristic constituent of this plant, exhibited a significant inhibitory effect on EBV-EA activation. Furthermore, the cell cycle analysis of 2 on Raji cells was also examined and strong inhibition was observed on the effect of the cell cycle induced by TPA. Compound 2 showed potent anti-tumor-promoting activity in two-stage carcinogenesis tests of mouse skin using 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA or fumonisin B1 as a promoter. Consequently, these results suggest that majonoside-R2 (2) could be a valuable chemopreventive agent against chemical carcinogenesis.

Key words majonoside-R2; oocititol-type saponin; Panax vietnamensis; anti-tumor-promoter; fumonisin B1; cell cycle analysis

The roots or rhizomes of many kinds of Panax plants (such as Panax ginseng, P. notoginseng, P. quinquefolium and P. japonicus) have been widely used for the treatment of many serious diseases and for enhancing physical strength in many countries.2 Cancer currently remains a very serious disease and is one of the major causes of death in the world, despite the fact that many kinds of antitumor agents have been isolated from natural resources and medicinal sciences have made rapid progress in the treatment of cancer. Further, the ideal effective antitumor agent free from any side-effects has not been found yet, and such side-effects are serious and critical problems in treating cancer. Therefore, progress in cancer chemoprevention is extremely important as is the development of cancer treatment. In the multi-stage theory of chemical carcinogenesis, the development of inhibitors of the promotion stage has been regarded as the most effective method for the chemoprevention of cancer.3

In our search for novel cancer chemopreventive agents (anti-tumor-promoters) from natural sources, we have carried out a two-stage carcinogenesis test on many kinds of natural products (triterpenoids,4 flavonoids,5 euglobals6) and kampo prescriptions7) using their inhibitory effects on mouse skin tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as a promoter. In the course of our continuing search for cancer chemopreventive agents, several kinds of Panax plants were screened and consequently, P. notoginseng exhibited strong anti-tumor-promoting activity on the two-stage carcinogenesis of mouse skin and pulmonary tumors.8 Also, the crude saponin fraction of Panax vietnamensis Ha et Grushv. exhibited strong inhibitory effects in the primary in vitro screening test using Epstein-Barr virus early antigen (EBV-EA) activation induced by TPA. P. vietnamensis is a new Panax species discovered in Central Vietnam in 1973, and the roots and rhizomes of this plant has been used secretly by the Sedang ethnic minority as a life-saving plant drug. Many kinds of new dammarane saponins had been isolated together with known ginsenosides similar to those in P. ginseng, and their chemical investigation has been reported.9 Moreover, the extremely high yield of dammarane saponins having an oocititol side-chain, especially majonoside-R2 (2) (more than 5% and ca. half the total yield of saponin), has made P. vietnamensis an interesting crude drug of the Panax species.10 Furthermore, the effects of 2 on pentobarbital sleep and gastric lesions have also been reported.11

In this paper, on the anti-tumor-promoting activity of several kinds of saponins (1—7) isolated from P. vietnamensis (Chart 1), we report the results of the primary in vitro screening test on EBV-EA activation and the cell cycle analysis on Raji cells. The results of the in vivo two-stage carcinogenesis tests on mouse skin tumor promotion induced by two different types of promoters (TPA and fumonisin B112) of majonoside-R2 (2) are also reported.

MATERIALS AND METHODS

Plant Material and Extraction The material for this investigation, the roots and rhizomes of Panax vietnamensis Ha et Grushv., was collected in Gia-Lai-Kontum Province, Cetral Vietnam in August 1978, and herbarium specimens were deposited at the Ho Chi Minh University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam. The MeOH extract (yield: 47% from dried roots and rhizomes) and the crude saponin fraction (81.5 g from 200 g of MeOH extract) were prepared by the previously reported method.10 The compounds [majonoside-R1 (1): 0.14%, -R2 (2): 5.29%, ginsenoside-Rb1, (3): 2.0%, -Rb2, (4): 0.01%, -Rd (5): 0.87%, -Re (6): 0.17% and -Rg1 (7): 1.37% of the dried material, respectively], as shown in Chart 1, were isolated from this crude saponin fraction, and all compounds were identified by their physicochemical data.9

Chemicals The cell culture reagents, n-butyric acid and

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other reagents, were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA, 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 conditions described previously. 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. (Hamamatsu, Japan), and the animals were housed, 5 per polycarbonate cage, in a temperature-controlled room at 24±2°C and given food and water ad libitum.

**In vitro EBV-EA Activation Experiments** Inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer), the EBV genome-carrying human lymphoblastoid cells, cultured in 10% FBS RPMI 1640 medium. The indicator cells (Raji, 1×10⁶/ml) were cultured at 37°C for 48 h in 1 ml medium containing n-butylcytosine (4 mmol, inducer), TPA (32 pmol=20 ng in dimethylsulfoxide (DMSO), 2 µl), and various amounts of test compounds dissolved in 5 µl 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. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was expressed by comparison with that of the positive control experiment (100%) which was carried out with n-butylcytosine (4 mmol) plus TPA (32 pmol). In the experiments, EBV-EA induction was ordinarily around 35% and this value was taken as the positive control (100%). Four millimolar n-butylcytosine alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the trypan-blue staining method.

**Cell Cycle Analysis by 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 (1×10⁶/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. Nonionic detergent Triton X100 (0.1%) was added to the tubes to lyse the cell membrane. Treated Raji cells were passed through a 37 µm-pore nylon filter (Kyoshin Riko Co., Ltd., Tokyo, Japan) before staining. RNase in phosphate-buffered saline (PBS) (final concentration, 0.1%) was used to reduce the fluorescence intensity of RNA. Finally, propidium iodide (final: 50 µg/ml) was used to stain viable DNA. The flow cytometric analysis was carried out with an FAC Scan cell fit DNA system, and the cell cycle pattern was analyzed using its program.

**In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Promoted by TPA** 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 topicaly treated with DMBA (100 µg, 390 nmol) in acetone (0.1 ml) as an initiation treatment. One week after this initiation, papilloma formation was promoted twice a week by the application of TPA (1 µg, 1.7 nmol) in acetone (0.1 ml) to the skin. Group I received this TPA treatment alone, and groups II and III received a topical application of majonoside-R2 (2, 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 monitored weekly for 20 weeks, as described previously.

**In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Promoted by Fumonisin B1** The animals were divided into two experimental groups, 15 mice each. Initiation with DMBA was carried out by the same method as described above. One week after initiation, papilloma formation was promoted twice a week by the application of fumonisin B1 (51 nmol) in acetone (0.1 ml) to the skin. Group I received this fumonisin B1 treatment alone, and group II received a topical application of majonoside-R2 (2, 510 nmol) in acetone (0.1 ml) 1 h before each promotion treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

**RESULTS AND DISCUSSION**

The primary screening test was carried out using a short-term in vitro synergistic assay of EBV-EA activation induced by TPA. Although the inhibitory effect of the MeOH extract of *P. vietnamensis* (about 38% inhibition at 100 µg/ml) was
stronger than that of *P. ginseng*, but less than that of *P. notoginseng*; the inhibitory effects of the crude saponin fraction (100% and 63.8% inhibition at 100 μg/ml and 10 μg/ml, respectively) was stronger than the MeOH extract. Further, the fraction which consisted of 2 and 7 exhibited a stronger inhibitory effect than the crude saponin fraction, as shown in Table 1. Therefore, it was deduced that the active compound was present in the saponin fraction and primary screening of seven saponins (1–7) isolated from this active fraction was carried out.

As shown in Table 2, in this assay, all tested compounds exhibited inhibitory effects on EBV-EA activation without cytotoxicity on Raji cells, even at the highest concentration (2.5×10³ mol ratio/TPA). Of these saponins, majonoside-R1 (1) and R2 (2) exhibited a significant inhibitory effect on EBV-EA activation (100% inhibition at 1×10³ mol ratio/TPA and more than 40% inhibition was observed even at 1×10² mol ratio/TPA). In particular, compound 2 exhibited the most potent inhibitory effect (more than 80% and 50% inhibition were observed at 5×10² and 1×10² mol ratio/TPA, respectively). Their inhibitory effect was much greater than that of glycyrrhetic acid, which is known to be a potent anti-tumor-promoter. In our experiments, the inhibitory effect on EBV-EA activation correlates well with the anti-tumor-promoting activity in vivo. Therefore, the major saponin of *P. vietnamensis*, majonoside-R2 (2), may be anti-tumor-promoter.

The effects of 2 on the cell cycle of Raji cells treated with TPA were examined by flow cytometry. As shown in Table 3, the promoter TPA increased the percentage of the G₂ phase (34.7%) of the Raji cell cycle and reduced the percentages of both G₁ and S phases (56.3 and 9.0%, respectively) in comparison with those of the negative control cultivated without TPA and n-butyrice acid. Following treatment with majonoside-R2 (2), the percentage of the S phase of Raji cells increased but the percentage of the G₂ phase was reduced at any concentration of 2, compared with the positive control treated with TPA and n-butyrice acid. Accordingly, it was deduced that 2 may accumulate in Raji cells during the S phase, depending on the concentration, which may result in restoration of the percentage of the G₂ phase at the normal value in the negative control. Therefore, it was concluded that 2 strongly inhibits one of the biological activities of the promoter TPA by influencing the cell cycle.

Table 1. Percentages of EBV-EA Induction in the Presence of Extracts and Fractions of *Panax vietnamensis* with Respect to the Positive Control (100%)

<table>
<thead>
<tr>
<th></th>
<th>Concentration (μg/ml)²¹</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>P. vietnamensis</em> MeOH extract</td>
<td>62.1±3.4 (60)³⁰</td>
</tr>
<tr>
<td>Crude saponin fraction</td>
<td>0.0±0.6 (60)</td>
</tr>
<tr>
<td>GinsenosideRg₁ + majonoside-R2 fraction</td>
<td>0.0±0.3 (60)</td>
</tr>
</tbody>
</table>

a) TPA concentration was 20 ng (32 pmol)/ml. b) Values represent percentages relative to the positive control value (n=3, and ±S.D.). c) Values in parentheses are viability percentages of Raji cells; unless otherwise stated, the viability percentages of Raji cells were more than 80%. d) This fraction was prepared by elution column chromatography on Diaion HP-20 of the MeOH extract of *P. vietnamensis* with MeOH. e) This fraction was prepared from the crude saponin fraction by column chromatography on RP-8 and HPLC on RP-18.

Table 2. Percentages of EBV-EA Induction in the Presence of Saponins (1–7) with Respect to the Positive Control (100%)

<table>
<thead>
<tr>
<th></th>
<th>Concentration (mol ratio/TPA)²³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5×10¹</td>
</tr>
<tr>
<td>Majonoside-R1 (1)</td>
<td>0.0±0.0 (60)</td>
</tr>
<tr>
<td>Majonoside-R2 (2)</td>
<td>0.0±0.0 (60)</td>
</tr>
<tr>
<td>Ginsenoside-Rb₁ (3)</td>
<td>0.0±0.3 (80)</td>
</tr>
<tr>
<td>Ginsenoside-Rb₂ (4)</td>
<td>0.0±0.2</td>
</tr>
<tr>
<td>Ginsenoside-Rd (5)</td>
<td>0.0±0.0 (80)</td>
</tr>
<tr>
<td>Ginsenoside-Re (6)</td>
<td>0.0±0.2</td>
</tr>
<tr>
<td>Ginsenoside-Rg₁ (7)</td>
<td>0.0±0.0 (80)</td>
</tr>
</tbody>
</table>

a) TPA (32 pmol=20 ng/ml). b) Values represent percentages relative to the positive control values (n=3, and ±S.D.). c) Values in parentheses are viability percentages of Raji cells; unless otherwise stated, the viability percentages of Raji cells were more than 80%. d) not tested at these concentrations.

Table 3. Flow Cytometric Analysis of the Raji Cell Cycle Treated with Majonoside-R2 (2)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Positive control</th>
<th>Medium only</th>
<th>Treated with comp. 2 (nmol)³¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>G₁</td>
<td>56.3±1.2</td>
<td>60.5±1.0</td>
<td>57.3±2.1</td>
</tr>
<tr>
<td>S</td>
<td>9.0±0.5</td>
<td>28.2±0.8</td>
<td>12.2±1.6</td>
</tr>
<tr>
<td>G₂+M</td>
<td>34.7±0.9</td>
<td>11.3±0.9</td>
<td>30.5±1.2</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

a) Raji cells were treated with TPA (32 pmol) and n-butyrice acid. b) Raji cells cultivated in RPMI-1640 medium (1 ml) containing 10% fetal calf serum. c) Treated with TPA (32 pmol), n-butyrice acid, and 2 (32, 16 and 3.2 nmol are equal to 1000, 500 and 100 mol ratio/TPA). The values of 2 on the S and G₂+M phases at 32.0 and 16.0 nmol were significantly different from the positive control values (±S.D. and p<0.05).
Fig. 1. Inhibition of TPA-Induced Tumor Promotion Following Multiple Application of Majonoside-R2 (2) 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 2; □, TPA + 85 nmol of glycyrrhetic acid. At 10 and 15 weeks of promotion, the group treated with compound 2 was significantly different from the positive control group (p<0.05) in terms of papilloma bears (%), and, at 20 weeks of promotion, the group treated with compound 2 was different from the control group (p<0.05) in terms of papillomas per mouse (n=15 and, at 20 weeks of promotion, positive control group: 9.1±1.1, treated group: 4.9±0.9).

Fig. 2. Inhibition of Fumonisin B1-Induced Tumor Promotion Following Multiple Application of Majonoside-R2 (2)

All mice were initiated with DMBA (390 nmol) and promoted with fumonisin B1 (51 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 + 510 nmol of 2. At 10 and 15 weeks of promotion, the group treated with compound 2 was significantly different from the positive control group (p<0.05) in terms of papilloma bears (%), and, at 20 weeks of promotion, the group treated with compound 2 was different from the control group (p<0.05) in terms of papillomas per mouse (n=15 and, at 20 weeks of promotion, positive control group: 8.7±0.7, treated group: 6.2±0.8).

On the basis of the results of the in vitro assays described above, we investigated the inhibitory effect of 2 on the two-stage carcinogenesis test of mouse skin using DMBA as an initiator and TPA as a promoter. The incidence (%) of papilloma-bearing mice and the average number of papillomas per mouse are presented in Figs. 1A and 1B, respectively.

The incidence of papillomas in the positive control group was highly significant in 100% of the mice after 9 weeks of promotion. Further, more than 4 and 9 papillomas were formed per mouse after 10 and 20 weeks of promotion, respectively. When majonoside-R2 (2) was applied before each TPA treatment, the formation of papillomas in mouse skin was delayed and the mean number of papillomas per mouse was reduced; only about 30, 50 and 80% of the mice bore papillomas after 10, 15 and even 20 weeks of promotion, respectively, and less than 1 and 5 papillomas were formed per mouse after 10 and 20 weeks of promotion, respectively. The inhibitory effect of 2 on two-stage carcinogenesis was stronger than that of other tested triterpenoids, soyasaponin I, ginsenoside Rg1, 23,24-dihydrcocurbinac F and its glycoside, whose effects have been reported. Also, as shown in Fig. 1, the inhibitory effect of 2 on two-stage carcinogenesis is greater than that of glycyrrhetic acid.

In addition, fumonisin B1 has recently been studied as a new and non-TPA type tumor-promoter. It is one of the mycotoxins produced by Fusarium moniliforme, a common mold associated with corn. This mycotoxin exhibited strong promoting activity in an in vivo carcinogenesis test initiated with DMBA, although, in vitro, it did not show any EBV-EA activation, induction of ornithine decarboxylase activity, pro-
tein kinase C activity or enhancement of phospholipid synthesis, unlike TPA.  

The inhibitory effect of majoniside-R2 (2) on the two-stage carcinogenesis promoted by fumonisins B1 was also investigated, and the results are shown in Figs. 2A and 2B.

In the positive control group, all the mice bore papillomas after 11 weeks of promotion and more than 6 and 9 papillomas were formed per mouse after 15 and 20 weeks of promotion, respectively. When compound 2 was applied, the papilloma formation was markedly delayed (only less than 30 and 70% of mice bore papillomas after 11 and 20 weeks of promotion, respectively), and the number of papillomas per mouse was reduced (only about 4 and 6 papillomas were formed per mouse after 15 and 20 weeks of promotion, respectively) compared with the positive control. Therefore, majoniside-R2 (2) also apparently inhibits the tumor-promotion induced by the non-TPA type promoter, fumonisins B1.

These in vivo results suggest that majoniside-R2 might be valuable as an anti-tumor-promoter and chemopreventive agent in chemical carcinogenesis. Moreover, on the basis of the extremely high yield of this compound, the cultivation of this plant, P. vietnamensis, might be valuable as a source of this chemopreventive agent.

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REFERENCES AND NOTES

1) A part of this work was presented at the International Symposium on Natural Medicines, Kyoto, October 1997, Abstracts Papers, p. 168.


15) Percentages of EBV-EA induction in presence of MeOH extract of P. gingivae and P. notoginseng were 79.6% (at 100 µg/ml) and 100% (at 10 µg/ml) and 7.6% (at 100 µg/ml) and 53.8% (at 10 µg/ml), respectively.

16) A high viability of Raji cells is necessary for in vitro assay using an indirect immunofluorescence technique by antigen-antibody reaction and is beneficial for the following in vivo assay.

17) Percentages of EBV-EA induction in presence of glycyrrhizic acid was 15.6, 54.3, 100 and 100% at 1×10⁻³, 5×10⁻³, 1×10⁻² and 1×10⁻¹ mol ratio/TPA, respectively and the viability percentage of Raji cells was more than 80% at each concentration. Tokuda H., Ohgishi H., Koshimizu K., Ito Y., Cancer Lett., 33, 279—282 (1986); Mizutani K., “Food Phytochemicals for Cancer Chemoprevention II, Teas, Spices, and Herbs,” ed. by Ho C.-T., Osawa T., Huang M.-T., Rosen R.T., American Chemical Society, Washington, D.C., 1994, pp. 322—328.
