Cancer chemopreventive activity of flavanones on Epstein–Barr virus activation and two-stage mouse skin carcinogenesis

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Received 9 August 2001; received in revised form 24 August 2001; accepted 24 August 2001

Abstract

To search for possible cancer chemopreventive agents from natural sources, we performed primary screening of ten flavanones isolated from plants belonging to Rutaceae and Leguminosae by examining their possible inhibitory effects on Epstein–Barr virus (EBV) early antigen activation induced by 12-\textit{O}-tetradecanoylphorbol-13-acetate in Raji cells. All of the flavanones tested in this study showed inhibitory activity against EBV, without showing any cytotoxicity. Amorilin (3), which has three prenyl (3-methyl-2-butenyl) side-chains in the molecule, showed the most potent activity. Furthermore, lupinifolin (5) exhibited a marked inhibitory effect on mouse skin tumor promotion in an in vivo two-stage carcinogenesis test. These results indicate that some of these prenylated flavanones might be valuable as potential cancer chemopreventive agents (anti-tumor promoters). © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cancer chemopreventive agents; Anti-tumor-promoting effect; Epstein–Barr virus activation; Two-stage mouse skin carcinogenesis; Flavanones

1. Introduction

The recently introduced practice of chemoprevention is dedicated to identifying agents with potential preventive roles in cancer. It is generally accepted that carcinogenesis is a complex and multistage process involving initiation, promotion and progression steps. Natural products which block the tumor-promoting step, a long and reversible process in multistage carcinogenesis, have been vigorously sought in recent years. Since the advent of chemopreventive studies, short-term in vitro models used in the study of carcinogenesis have been applied for the identification of anti-tumor agents. The Epstein–Barr virus (EBV) is known to be activated by tumor promoters, including 12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA), to produce early antigens (EA). Evaluation of the Epstein–Barr virus early antigen (EBV-EA) inhibition is now used as a primary in vitro screening for anti-tumor-promoting activities. We have previously reported some cancer chemopreventive agents by this short-term in vitro assay [1–7]. The compounds effective in
this assay also showed an in vivo effect against skin carcinogenesis [1–3].

As active flavonoids, polymethoxyflavonoids [1], isoflavonoids [2] and biflavonoids [4] have been isolated from Rutaceae, Leguminosae and Guttiferae plants, respectively. Strong cancer preventive potential was found in those constituents with a prenyl side-chain in phenylpropanoid [3], 7-methoxycoumarin [5], xanthone [6] and carbazole [7] molecules. Furthermore, we have reported in series that the isoflavonoids, the prenyl group-carrying components of Leguminosae, were promising sources of effective chemopreventive agents [2]. In this study, we investigated the inhibitory effects of flavanones, a different flavonoid class with a different skeleton isolated from Rutaceae (five Citrus) and Leguminosae (Sophora, Amorpha and Derris), on EBV-EA activation and in vivo two-stage mouse skin carcinogenesis.

2. Materials and methods

2.1. Test products

The chemical structures and the plant sources of all flavanones are shown in Fig. 1 and Table 1, respectively. The purity of each of the compounds tested was corroborated by measurements of melting points, IR, MS and 1H-NMR spectra.

2.2. In vitro EBV-EA activation experiment

The inhibition of EBV-EA activation was assayed using the same method as has been described previously [1–7]. Raji cells (10^6 cells/ml) were incubated at 37°C for 48 h in RPMI 1640 medium with 10% fetal calf serum (FCS) containing n-butyric acid (4 mmol), TPA (32 pmol), and various amounts of test compound. Smears were made from the cell suspension, and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique. In each assay, at least 500 cells were counted and the number of stained cells (positive cells) was recorded. Each assay was repeated three times for one test compound. The EBV-EA-inhibiting activity of the test compound was estimated on the basis of the percentage of the number of positive cells compared with that of the control without the test compound. The viability of the cells was assayed by the Trypan-Blue staining method. For the determination of cytotoxicity, the cell viability was required to be more than 60%.

2.3. In vivo two-stage mouse skin carcinogenesis test

A total of 30 female ICR mice (6 weeks old, purchased from SLC Co. Ltd., Shizuoka, Japan) were used. Two groups, with each group consisting of 15 animals, housed at five/cage, were painted with 390 nmol of 7,12-dimethylbenz[a]anthracene (DMBA) in
Table 1
Inhibitory effects of flavanones on TPA-induced EBV-EA activationa

<table>
<thead>
<tr>
<th>Compound</th>
<th>EBV-EA-positive cells (% viability)</th>
<th>Plantb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compound concentration (mol ratio/32 pmol TPA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>Sophoraflavone-B (1)</td>
<td>0.0 ± 0.2 (70)</td>
<td>30.0 ± 1.4 (&gt;80)</td>
</tr>
<tr>
<td>Senegalensein (2)</td>
<td>0.0 ± 0.5 (70)</td>
<td>19.9 ± 1.2 (&gt;80)</td>
</tr>
<tr>
<td>Amorilin (3)</td>
<td>0.0 ± 0.4 (70)</td>
<td>16.4 ± 1.5 (&gt;80)</td>
</tr>
<tr>
<td>Euchrestaflavone-A (4)</td>
<td>0.0 ± 0.6 (70)</td>
<td>23.8 ± 2.3 (&gt;80)</td>
</tr>
<tr>
<td>Lupinifolinol (5)</td>
<td>0.0 ± 0.4 (60)</td>
<td>30.1 ± 1.3 (&gt;80)</td>
</tr>
<tr>
<td>Lupinifolinol (6)</td>
<td>0.0 ± 1.1 (60)</td>
<td>26.3 ± 2.0 (&gt;80)</td>
</tr>
<tr>
<td>Paratocarpin-I (7)</td>
<td>0.0 ± 0.7 (70)</td>
<td>31.1 ± 1.8 (&gt;80)</td>
</tr>
<tr>
<td>Erythrisenegalone (8)</td>
<td>0.0 ± 0.3 (70)</td>
<td>32.2 ± 2.0 (&gt;80)</td>
</tr>
<tr>
<td>Citiflavone (9)</td>
<td>12.6 ± 0.6 (70)</td>
<td>47.3 ± 1.3 (&gt;80)</td>
</tr>
<tr>
<td>Yukovanol (10)</td>
<td>10.1 ± 0.3 (70)</td>
<td>41.7 ± 1.6 (&gt;80)</td>
</tr>
</tbody>
</table>

a Values are EBV-EA activation (%) ± SD in the presence of the test compound relative to the positive control (100%). Activation was attained by treatment with TPA (32 pmol/ml). Values in parentheses represent the viability percentage of Raji cells, as measured by Trypan-Blue staining. At least 60% viability of Raji cells 2 days after treatment with the compounds is required for an accurate result.
b, a, Sophora tomentosa L. (Leguminosae); b, several hybrid seedlings resulting from a cross of Citrus tamurana Hort. ex. Takahashi × Citrus kinokuni Hort ex. Tanaka (Rutaceae); c, Amorpha fruticosa L. (Leguminosae); d, Bor Tenga (this is a citrus plant cultivated in India, and considered to be a cultivar derived from C. grandis; Rutaceae); e, Citrus medica L. var. etrog Engl. (Rutaceae); f, Derris trifoliata Lour. (Leguminosae); g, Citrus sinensis Osbeck (Rutaceae); h, Citrus yuko Hort. ex. Tanaka (Rutaceae).

3. Results and discussion

The anti-tumor-promoting activity of each of ten natural flavonoids, including flavanones (1–5 and 7–9) and flavanonols (6 and 10), was tested in a short-term in vitro assay of TPA-induced EBV-EA activation in Raji cells. Their inhibitory effects on the activation of the virus-genome, and the viability of the Raji cells, are shown in Table 1. All flavonoids tested showed an inhibitory effect on EBV activation, even at a 1 × 10 mol ratio. Only weak cytotoxicity against Raji cells was observed for all compounds, even at a concentration of 1 × 10 mol ratio.

Eight flavanoids (1–8) having a prenyl (3-methyl-2-butenyl) side-chain in the molecule were found to show 100% inhibitory activity at 1 × 10 mol ratio. However, the inhibitory activities of compounds lacking a prenyl group (9 and 10) were not fully estimated (87.4–89.9% inhibition of activation at 1 × 10 mol ratio/TPA). Among all of the prenylated flavanones tested in the present study, amorilin (3), with three prenyl groups in the molecule, exhibited the most potent inhibitory activity (100% inhibition of activation at 1 × 10 mol ratio/TPA, and 83.6, 39.7 and 18.0% inhibition of activation at 5 × 10, 1 × 10 and 1 × 10 mol ratio/TPA, respectively). Therefore, the inhibitory activity was shown to be correlated with the number of prenyl groups in the molecule. The inhibitory activity of senegalensein (2) and euchrestaflavanone-A (4), with two prenyl side-chain groups showed more potency than that of the compounds (1 and 5–8) with one prenyl group in the molecule. On the other hand, comparing the flavanones (5 and 9) and flavanonols (6 and 10), the presence of a hydroxy group at C-3 is not essential for the activity.
In previous papers, we reported that the presence of the prenyl side-chain on phenylpropanoid, 7-methoxycoumarin, xanthone or carbazole nuclei plays an important role in anti-tumor-promoting activity [3,5–7]. Furthermore, isoflavonoids having more than one prenyl side-chain in the molecule exhibit even greater potency [2]. Therefore, from the viewpoint of structure–activity relationships, an essential feature for the activity of flavanones examined in the present study is considered to be the presence of the prenyl side-chain in the molecule.

Based on the results obtained in vitro, the inhibitory effect of lupinifolin (5), a major component of Derris trifoliata Lour. (Leguminosae), was investigated in an in vivo two-stage carcinogenesis test focusing on mouse skin papillomas induced by DMBA as an initiator and TPA as a promoter. The activity evaluated in terms of both the rate (%) of papilloma-bearing mice (Fig. 2A) and the average number of papillomas/mouse (Fig. 2B) was compared with that of a positive control. In the case of the positive control, 33, 80 and 100% of the mice bore papillomas after 6, 8 and 10 weeks of promotion, respectively, and more than 9.7 papillomas were formed/mouse after 20 weeks, as shown in Fig. 2. When lupinifolin (5) was applied before TPA treatment, it delayed the formation of papillomas as follows. In the group treated with lupinifolin (5), only about 20 and 60% of mice bore papillomas after 10 and 15 weeks of promotion, respectively, and only 93% of the mice bore papillomas, even after 20 weeks. Also lupinifolin (5) reduced the number of papillomas/mouse as follows. Less than 2.1 and 3.2 papillomas were formed/mouse after 12 and 17 weeks of promotion, respectively, and only about 4.3 papillomas were formed/mouse even after 20 weeks, as shown in Fig. 2B.

At present, the mechanism included in tumor promoter-induced activation of EBV-EA is still not clear. One possibility would involve the activation of protein kinase C (PKC). TPA can activate PKC and the cellular receptor could be PKC itself [8,9]. The data from us and other groups have demonstrated that the results from EBV-EA assays well correlate with that in vivo anti-tumor effect. A study of ten natural flavanones on EBV-EA activation demonstrated that consistent with previously reported isoflavonoids [2], the presence of one or more prenyl side-chains in the molecule is an important structural char-

![Fig. 2. Inhibitory effects of lupinifolin (5) on DMBA–TPA mouse skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas/mouse. (●) Control TPA alone; (○), TPA + 85 nmol of lupinifolin (5). After 20 weeks of promotion, a significant difference in the number of papillomas/mouse between the groups treated with compound 5 and the control group was evident ($P < 0.05$).]
acteristic for the inhibitory effect of flavonones. Flavanones effective in the EBV-EA assay were also effective in vivo against two-stage mouse skin carcinogenesis, suggesting that they could be potential as human cancer chemopreventive agents.

Acknowledgements

This investigation was partly supported by Grants-in-Aid for Scientific Research (C) (H.F., number 09672173) and a High-Tech Research Center Project from the Japan Society for the Promotion of Science and The Ministry of Education, Science, Sports and Culture of Japan, respectively. This study was also supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, and the Ministry of Health and Welfare, Japan (H.N.).

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