Flavoglaucin, its derivatives, and pyranonigrins, which are antioxidants produced by the molds used in fermented foods, were examined for their inhibition of tumor promotion by the Epstein-Barr virus early antigen activation test. Flavoglaucin and its derivatives exhibited high activity. Flavoglaucin and such a derivative as isodihydroauroglaucin inhibited mouse skin tumor promotion in a two-stage carcinogenesis test and appear to be antitumor promoters.

Key words: antitumor promoter; Epstein-Barr virus activation; flavoglaucin; mold; two-stage skin carcinogenesis

Cancer chemoprevention to inhibit tumor promotion has been regarded as one means of cancer control; it has been the focus of attention because tumor promotion takes a long time to occur and is the only reversible process during the multiple stages of carcinogenesis.\(^1\)\(^2\) The Epstein-Barr virus early antigen (EBV-EA) activation test is known to be a convenient \textit{in vitro} assay for detecting naturally occurring antitumor promoters. Effective compounds have also been evaluated \textit{in vivo} for their inhibiton of tumor promotion by using the EBV-EA activation test \textit{in vitro} and a two-stage mouse skin carcinogenesis test.\(^3\)

Traditional Japanese fermented foods, including rice wine (sake), distilled spirit (shochu), soy paste (miso), soy sauce (shoyu), and dried bonito (katsuobushi), are manufactured by fermentation, using molds of such filamentous fungi as the \textit{Aspergillus} and \textit{Eurotium} species. These molds are thought to help protect fermented foods from oxidative deterioration by producing secondary metabolites having antioxidative activity or by converting some ingredients in the food to potent antioxidants.\(^4\)\(^5\) These molds have been used for manufacturing fermented food for many years and have been authorized to be generally recognized as safe (GRAS). We have reported on isolated antioxidants, including such pyranonigrins as pyranonigrin-A and pyranonigrin-S from \textit{Aspergillus kawachii}, which are used in shochu production, and flavoglaucin and such derivatives as tetrahydroauroglaucin, dihydroauroglaucin, isodihydroauroglaucin, and auroglaucin from \textit{Eurotium herbariorum} which are used in producing katsuobushi (Fig. 1).\(^6\)\(^7\) These compounds have been shown to have high scavenging activity for the superoxide radical and are expected to possess other bioactivity.\(^5\)\(^7\) The antioxidants in the present study were isolated from molds of fermented foods and examined for their inhibition of tumor promotion by using the EBV-EA activation test \textit{in vitro} and a two-stage mouse skin carcinogenesis test \textit{in vivo}.

The inhibition of EBV-EA activation was assayed by using the method described previously.\(^8\) Raji cells were grown to a density of 10^6 cells/ml, harvested by centrifugation and resuspended in an RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) with 10% FCS containing 4 mM n-bytyric acid as inducer, 32 pmol of 12-O-tetradecanoylphorbol-13-acetate (TPA), and 32, 16, 3.2 or 0.32 nmol of the test compound (a DMSO solution). Each DMSO sample solution was put into an assay tube and incubated at 37°C for 48 h, and the cell number and viability were determined after 48 h with a hemocytometer by the Trypan Blue staining method. A minimum 60% survival rate of the Raji cells after treating with the compounds was required for an accurate result. The EBV-EA inhibitory activity of each test compound was estimated on the basis of the percentage of positive cells compared to that observed in a control without the test compound. The IC\textsubscript{50} value in each assay was estimated by the probit transformation technique.

The IC\textsubscript{50} values for the EBV-EA inhibitory activity of flavoglaucin, its derivatives (tetrahydroauroglaucin, dihydroauroglaucin, isodihydroauroglaucin, and auroglaucin), and pyranonigrins (pyranonigrin-A and pyranonigrin-S) are shown in Table 1. A minimum 60% survival rate of the Raji cells is shown for samples with a concentration of less than 1,000 (mol ratio/32 pmol TPA). A comparison of the IC\textsubscript{50} values shows that flavoglaucin and its derivatives exhibited a higher inhibitory effect for TPA-induced EBV-EA activation.
Flavoglaucin and isodihydroauroglaucin have been reported to be produced as the main compounds by Eurotium herbariorum NE-1, a typical filamentous fungus used for katsuobushi production. We attempted to examine flavoglaucin and isodihydroauroglaucin for their antitumor promoting activity in vivo with a two-stage mouse skin carcinogenesis test by using the method described previously. Female SENCAR mice were obtained at 5–6 weeks of age from SLC Co. (Shizuoka, Japan). Groups of animals (15 animals per group) were housed in subgroups of five in polycarbonate cages. The mice were permitted free access to an MF solid diet (Oriental Yeast Co., Chiba, Japan) and drinking water at all times during the study. The back of each mouse was shaved with surgical clippers before the first day of initiation. A tumor was initiated on the back of each mouse with dimethylbenz[a]anthracene (DMBA; 390 nmol) in acetone (0.1 ml). One week after initiation, the tumor was promoted twice a week by applying TPA (1.7 nmol) in acetone (0.1 ml). The mice in the groups treated with a test compound were applied with the compounds (85 nmol) in acetone (0.1 ml) for 1 h before each TPA treatment. The incidence of papillomas was observed than curcumin, a typical antitumor promoter, although the pyranonigrins showed weak activity. Flavoglaucin and its derivatives exhibited this higher inhibitory effect than curcumin at 10 and 100 levels (mol ratio/32 pmol TPA), although they had lower activity at the 500 level (mol ratio/32 pmol TPA). These compounds thus possessed their stronger activity than curcumin at low concentrations. There was not much difference in IC$_{50}$ values between flavoglaucin and its derivatives (280–299 mol ratio/32 pmol TPA). These results indicate flavoglaucin and its derivatives (tetrahydroauroglaucin, dihydroauroglaucin, isodihydroauroglaucin, and auroglaucin) to be effective inhibitors of tumor promotion in vitro. The number and position of the double bond in the side chain of hydrocarbon at the C-6 position on benzohydroquinone seems not to have been important for heigh activity. We have reported that the prenyl group on phenylpropanoids, cinnamylphenol, and terpenoid coumarins was related to the inhibitory activity of tumor promotion by the EBV-EA activation test. We consider that the prenyl group at the C-3 position on flavoglaucin and its derivatives was related to the expression of activity in a similar manner to the reported compounds.

### Table 1. Ratio (%) of EBV-EA Positive Cells by Compounds in the EBV-EA Activation Test and Their IC$_{50}$ Values (mol ratio of TPA)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound concentration (mol ratio/32 pmol TPA)$^a$</th>
<th>IC$_{50}$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>Flavoglaucin</td>
<td>0 ± 0.2$^e$ (60$^f$)</td>
<td>28.5 ± 0.6 (&lt;80)</td>
</tr>
<tr>
<td>Tetrahydroauroglaucin</td>
<td>0.0 ± 0.3 (60)</td>
<td>28.9 ± 0.5 (&lt;80)</td>
</tr>
<tr>
<td>Dihydroauroglaucin</td>
<td>0 ± 0.2 (60)</td>
<td>27.3 ± 0.5 (&lt;80)</td>
</tr>
<tr>
<td>Isodihydroauroglaucin</td>
<td>0 ± 0.2 (60)</td>
<td>28.0 ± 0.6 (&lt;80)</td>
</tr>
<tr>
<td>Auroglaucin</td>
<td>0 ± 0.2 (60)</td>
<td>27.0 ± 0.5 (&lt;80)</td>
</tr>
<tr>
<td>Pyranonigrin-A</td>
<td>10.3 ± 0.5 (60)</td>
<td>47.3 ± 0.8 (&lt;80)</td>
</tr>
<tr>
<td>Pyranonigrin-S</td>
<td>12.5 ± 0.6 (60)</td>
<td>49.1 ± 0.8 (&lt;80)</td>
</tr>
<tr>
<td>Curcumin$^c$</td>
<td>0 ± 0.2 (60)</td>
<td>22.8 ± 0.6 (&lt;80)</td>
</tr>
</tbody>
</table>

$^a$Mol 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.

$^b$IC$_{50}$ represents the mol ratio of TPA that inhibited 50% of the positive control (100%).

$^c$Values (N = 3, mean ± SD) are EBV-EA activation (%) in the presence of test compounds relative to the control (100%) by measuring EBA-EA-positive cells.

$^e$Value in parentheses represents the viability (%) of Raji cells.

$^f$Positive control substance of a typical antitumor promoter.

Fig. 1. Chemical Structures of Flavoglaucin, Its Derivatives, and Pyranonigrins.
weekly for 20 weeks. The animal experimental protocol was approved by Institute for Experimental Animals of Kanazawa University Advanced Science Research Center.

Flavoglaucin and isodihydroauroglaucin exhibited the inhibitory activity toward mouse skin tumor promotion in the in vivo two-stage carcinogenesis test shown in Fig. 2. Flavoglaucin and isodihydroauroglaucin had the equivalent efficacy. Flavoglaucin has been reported to have a suppressive effect on intestinal neoplasia from azoxymethane-induced intestinal carcinogenesis in rats.13) Flavoglaucin and its derivative were also found to have an antitumor promoting effect on mouse skin carcinogenesis in the present study. The results of the present study indicate that flavoglaucin and its derivatives, which are produced by the molds used in katsuobushi production, might be valuable antitumor promoters. 

Reactive oxygen species (ROS) are known to play an important role in mutagenesis and carcinogenesis, particularly in tumor promotion.14) ROS such as the superoxide radical are produced by polymorphonuclear neutrophils, macrophages, and non-phagocytic cells stimulated by TPA-type tumor promoters.15) Curcumin has been reported to exhibit an inhibitory effect on TPA-induced superoxide radical generation in differentiated HL-60 cells and to have an inhibitory effect on tumor promoter-induced oxidative stress via both interference with the infiltration of leukocytes into the inflammatory regions and inhibition of their activation in mouse skin.16) We have reported that flavoglaucin and its derivatives had high scavenging activity toward the superoxide radical.17) We conjecture that the compounds in the present study might have similar activity to that of curcumin, and we think that the compounds should be examined for this activity in future.

Fig. 2. Inhibitory Effects of Flavoglaucin and Isodihydroauroglaucin on DMBA-TPA Mouse Skin Carcinogenesis.

A tumor was initiated in each mouse with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly, starting one week after initiation. A, Percentage of mice with papillomas. B, Average number of papillomas per mouse. FG, flavoglaucin; IDAG, isodihydroauroglaucin. A significant difference in the number of papillomas per mouse was evident between the groups treated with flavoglaucin or isodihydroauroglaucin and the control group after 20 weeks of promotion (p < 0.05). Each value is presented as the mean ± SD.

References