Anti-tumor Promoting Activities of Natural Products. II.1) Inhibitory Effects of Digitoxin on Two-Stage Carcinogenesis of Mouse Skin Tumors and Mouse Pulmonary Tumors2)

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Two-stage carcinogenesis of mouse skin papillomas induced by 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), and mouse pulmonary tumors induced by 4-nitroquinoline-N-oxide (4NQO) and glycerol, were inhibited by digitoxin (1).

Keywords digitoxin; anti-tumor promoter; two-stage carcinogenesis

We previously5) reported the inhibitory effects of thirty steroids and their glycosides on the Epstein-Barr virus early antigen (EBV-EA) induction in Raji cells, induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), as a primary screening test for anti-tumor promoting agents. In this in vitro assay, cardiac steroids such as digiotoxigenin and strophanthidin, and their glycosides, i.e., digitoxin (1), cymarin, and ouabain showed strong activities. Especially, 1 and cymarin showed potent inhibitory effects even at a low concentration [mol ratio of steroid:TPA = 1:1 (each 32 pmol/ml)] and at this concentration, the activity of 1 (and also cymarin) was greater than that of other natural products previously tested.4) On the basis of the results of this in vitro assay (inhibitory effects on EBV-EA activation), the inhibitory effects of 1 on two-stage carcinogenesis tests in vivo, i.e., the two-stage carcinogenesis of mouse skin papillomas5) and mouse pulmonary tumors,6) were investigated.

Materials and Methods

Materials 7,12-Dimethylbenz[a]anthracene (DMBA), TPA, and 1 were obtained from Sigma Chemical Co. (U.S.A.). 4-Nitroquinoline-N-oxide (4NQO) and high grade glycerol were purchased from Wako Pure Chemical Industries (Osaka, Japan).

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.

Two-Stage Carcinogenesis Test on Mouse Skin Papillomas5) Each group of 15 mice was housed with 5 mice per cage and given water ad libitum. The back of each mouse was shaved with surgical clippers. The mice were initiated by treating them with DMBA (100 µg, 390 nmol) in acetone. One week after initiation, they were promoted twice a week by application of TPA (1 µg, 1.7 nmol) in acetone. One hour before each TPA treatment, the mice were treated with 1 (85 nmol) in acetone. The incidence of papillomas was observed weekly for 20 weeks.

Two-Stage Carcinogenesis Test on Mouse Pulmonary Tumors6) All mice were fed ad libitum with a diet of commercial rodent pellets and allowed either tap water or water containing glycerol (8%). A total of 75 mice were divided into five groups of 15 animals. Each experimental group received the following initiation/promotion treatments: (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 with 1 (0.25 mg/100 ml) solution, n = 15. The total dose of 1 was 0.04 mg/mouse/week.7,8)

Initiation 4NQO was dissolved in a mixture of olive oil and cholesterol (20:1). Dosage of 0.3 mg per mouse was given by a single subcutaneous injection at the starting time (groups III, IV, and V). As a control, the mice were given only water (group I) or only 8% glycerol solution (group II) during experiments.

Promotion Glycerol was dissolved in water (8%) and given as drinking water ad libitum. Five weeks after initiation, the promoting treatment with 8% glycerol solution (groups II and IV) or 8% glycerol solution containing 1 (0.25 mg/100 ml) (group V) was started and continued for 20 weeks. Other groups (I and III) were given tap water ad libitum. The intake of these solutions was measured twice a week.

Treatment of Animals The experimental groups were killed by cervical dislocation after 25 weeks. 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 conventional methods to allow histological study of the pulmonary tumors.

Results and Discussion

On the basis of our reported in vitro results,3) the inhibitory effects of 1 on two-stage carcinogenesis of mouse skin papillomas,5) using DMBA as an initiator and TPA as a promoter, was investigated. The activities, evaluated by both the rate (%) of papilloma-bearing mice (Fig. 1A) and the average number of papillomas per mouse (Fig. 1B), were compared with those of positive controls. As shown

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in Fig. 1A, when I was applied continuously before each TPA treatment, I delayed the formation of papillomas on mouse skin compared with the control experiment with TPA alone; the rate of papilloma-bearing mice was also reduced (about 20% reduction at 20 weeks). In addition, as shown in Fig. 1B, I also reduced to average number of papillomas per mouse (about 50% reduction even at 20 weeks). Next, the two-stage carcinogenesis test of I on mouse pulmonary tumors was also carried out, using 4NQO as an initiator and glycerol as a promoter.

As shown in Table I, both the total number of pulmonary tumors in 15 mice and the percentage of mice with tumors were considerably reduced (about 50% reduction in the percentage of mice with tumors after 25 weeks) by taking I together with the promoter (group V), compared with those of the positive controls (group IV). The mean intake of drinking water was 7.0, 7.1, 7.0, 6.7, and 6.4 ml/mouse/d in groups I to V, respectively, and no statistically significant difference was observed between groups. Further, the increase in the body weight of the treated mice was not affected by treatment with I. Cardiac glycosides such as I have been widely used in the therapy of congestive heart failure and of certain other heart ailments. These results strongly suggest that I might be valuable as an anti-tumor promoter in chemical carcinogenesis. The inhibitory mechanisms of I on the tumor promotion is now being studied.

References and Notes
7. The LD50 value of I in the mouse (oral administration) is 4950 μg/kg. The dose of I in the present experiment is 5.7 μg/mouse (mean body weight = ca. 30 g)/d and this dose is about 1/26 of that of the LD50 value.
9. Anti-tumor promoting activity of digoxigenin (= glycycone of I) seems to be less potent compared with I. Hence, the inhibitory effects of I on EBV-VCA activation was stronger than that of digoxigenin. Further, in the two-stage carcinogenesis test on mouse skin papillomas, digoxigenin moderately delayed the formation of papillomas and moderately reduced the average number of papillomas per mouse (about 30% reduction at 20 weeks) compared with I but did not reduce the rate of papilloma-bearing mice at 20 weeks compared with the controls.

Table I. Incidences of Pulmonary Tumors in Mice Treated with 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of tumors</th>
<th>Number of tumors per mouse</th>
<th>% of mice with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>I water</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II 8% glycerol</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III 4NQO + water</td>
<td>2</td>
<td>0.13</td>
<td>6.6</td>
</tr>
<tr>
<td>IV 4NQO +8% glycerol</td>
<td>45</td>
<td>3.0</td>
<td>100.0</td>
</tr>
<tr>
<td>V 4NQO +8% glycerol + I (0.25 mg/100 ml)</td>
<td>14</td>
<td>0.93</td>
<td>53.3</td>
</tr>
</tbody>
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