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Auraptene, a Citrus Coumarin, Inhibits 12-*O*-Tetradecanoylphorbol-13-acetate-induced Tumor Promotion in ICR Mouse Skin, Possibly through Suppression of Superoxide Generation in Leukocytes

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Coumarin-related compounds, auraptene and umbelliferone, have been isolated from the cold-pressed oil of *natsumikan* (*Citrus natsudaidai* HAYATA), and tested as inhibitors of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation in Raji cells. The 50% inhibitory concentration (IC_{50}) of auraptene (18 μM) was almost equal to that of genistein. Umbelliferone, which lacks a geranyloxyl group present in auraptene, was less active ($IC_{50}=450\ \mu M$). In a two-stage carcinogenesis experiment with 7,12-dimethylbenz[*a*]anthracene (topical application at 0.19 μmol) and TPA (topical application at 1.6 nmol) in ICR mouse skin, topical application of auraptene (at 160 nmol) significantly reduced tumor incidence and the numbers of tumors per mouse by 27% ($P<0.01$) and 23% ($P<0.05$), respectively. Auraptene at a concentration of 50 μM markedly suppressed superoxide (O_2^-) generation induced by 100 nM TPA in differentiated human promyelocytic HL-60 cells. Having no O_2^--scavenging potential, auraptene may inhibit the multicomponent NADPH oxidase system. Inhibition of intracellular hydroperoxide formation in differentiated HL-60 cells by auraptene was also confirmed by flow-cytometric analysis using 2′,7′-dichlorofluorescein diacetate as a fluorescence probe. Quantitative analyses using high-performance liquid chromatography showed the occurrence of auraptene not only in both the peels and sarcocarps of *natsumikan*, but also in those of *hassaku* orange (*C. hassaku*) and grapefruit (*C. paradisi*), and even in their bottled fresh juice form. These results indicate that auraptene is a chemopreventer of skin tumorigenesis, and implies that suppression of leukocyte activation might be the mechanism through which it inhibits tumor promotion.

Key words: Auraptene — Citrus coumarin — Chemoprevention — Anti-tumor promoter — Superoxide

Epidemiological surveys and animal experiments have demonstrated that ingestion of some constituents from vegetables and fruits may contribute to the reduction of cancer incidence in humans.[1,2] Since cancer therapy is not yet effective in all cases, cancer chemoprevention is considered as an attractive and promising avenue for cancer control.[3] β-Carotene, a major carotenoid occurring widely in green-yellowish vegetables and fruits, is one of the most extensively studied agents for chemoprevention on account of its cancer-preventive potency with low toxicity in various animal models, as well as epidemiological evidence.[4,5] Several clinical trials using β-carotene have been completed or are ongoing in the USA, e.g., The Physicians' Health Study, the Women's Health Study,[6] and the CARET study.[7] Unexpectedly, however, β-carotene failed to reduce cancer risk or mortality in a recent clinical study.[8] To date, no beneficial effects of β-carotene in terms of chemoprevention have been reported except for the case of the Linxian study.[9] It is evident that β-carotene does not exclusively represent the cancer-preventive potential of vegetables. Hence, there is a need to discover new types of chemopreventive agents by scrutinizing a diverse range of edible plants.

We regard the inhibition of tumor promotion (anti-tumor promotion) with food phytochemicals as a potentially useful strategy for chemoprevention[10] since tumor promotion, which takes a long-time to occur, is a reversible step in the multistage process of carcinogenesis.[11] Such a characteristic should be favorable for anti-tumor promotion as a measure for cancer control in humans. One of the well-known biological activities of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a representative tumor promoter, is the activation of Epstein-Barr virus (EBV), a herpesvirus causative for African Burkitt's lymphoma,[12] anaplastic nasopharyngeal carcinoma (NPC),[13] and probably also gastric cancer, in part.[14] We have developed a convenient *in vitro* assay, the inhibition of tumor promoter-induced EBV activation, for estima-

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tion of the anti-tumor-promoting properties of edible Japanese[15,16] and southeast Asian plants,[17,18] as well as for isolation of their active constituents.[19–22] Most inhibitors identified by this assay have been proven to be inhibitors of TPA-induced tumor promotion in mouse skin,[16,21,23,24] 4-nitroquinoline 1-oxide (4-NQO)-induced rat tongue carcinogenesis[25] and azoxymethane (AOM)-induced rat colonic aberrant crypt foci (ACF) formation (T. Tanaka *et al.*, manuscript in preparation).[26]

Citrus fruits are widely known to contain a variety of chemopreventive agents such as limonoids and their glucosides, which inhibit benzo[*a*]pyrene-induced forestomach and lung carcinogenesis in mice, TPA-induced skin tumor promotion in mice,[27] and 7,12-dimethylbenz[*a*]-anthracene (DMBA)-induced oral carcinogenesis in hamsters.[28] *d*-Limonene is an inhibitor of AOM-induced rat colonic ACF formation,[29] and DMBA-induced rat mammary carcinogenesis.[30] Citrus fruits also contain flavonoids[31] such as hesperidin, which inhibits 4-NQO-induced rat oral carcinogenesis[32] and AOM-induced rat colonic ACF formation.[33] Furthermore, we have recently reported that a glyceroglycolipid from the leaves of bitter orange (*Citrus hystrix*) possesses a strong inhibitory effect on TPA-induced skin tumor promotion.[21] In our continuing search for effective chemopreventive agents from edible plants, peel oil of *natsumikan* (*Citrus natsudaidai* HAYATA), which is commonly used in food additives or cosmetics in Japan, exhibited a marked inhibition of TPA-induced EBV activation. Here we describe the isolation and identification of the anti-tumor promoter from the peel oil of *natsumikan*, its anti-tumor-promoting activity in ICR mouse skin, and a possible mechanism of action. The occurrence of the active constituent in a wide variety of citrus and their juices is also reported.

MATERIALS AND METHODS

Chemicals TPA was obtained from Research Biochemicals International, Natick, MA. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco RBL, Grand Island, NY. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes, Inc., Leiden, The Netherlands. Cytochrome *c* was obtained from Sigma, St. Louis, MO. High-titer EBV early antigen (EA)-positive sera from NPC patients were the gift of Prof. Dr. Ohsato (Health Sciences University of Hokkaido). FITC-labeled anti-human IgG was obtained from Dako, Glostrup, Denmark. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka.

Animals and cells Female ICR mice (7 weeks old) were obtained from Japan SLC, Shizuoka. Human B-lymphoblastoid Raji cells and human promyelocytic leukemia HL-60 cells were the gifts of Prof. Dr. Ohsato (Health Sciences University of Hokkaido) and Prof. Dr. Sasaki (Kyoto University), respectively.

Isolation of the active constituents of *natsumikan* Whole *natsumikan* (4.8 kg, fresh wt.) harvested in 1994 in Wakayama Prefecture, Japan were extracted with an FMC In-line Citrus Juice Extractor (FMC Corporation, Chicago, IL) to give the cold-pressed oil (9.5 g). The oil thus obtained was fractionated while monitoring inhibition of EBV activation. The active compounds were purified by silica gel (*n*-hexane/ethyl acetate, stepwise method) and high-performance liquid chromatography (HPLC) (column: μBondasphere C_{18}, Waters, Milford, MA, 19×150 mm; elute, 90% methanol in water; flow rate, 7.0 ml/min; detection, UV_{254nm}) to give auraptene (240 mg, retention time 12.2 min) and umbelliferone (13 mg, retention time 5.1 min). Both agents were identified by means of spectroscopic analyses (ultraviolet, infrared, proton- and carbon-nuclear magnetic resonance, and mass spectra). Their spectral data coincided with those previously reported.[34,35] The purity of both compounds, determined by HPLC analysis, was 99% or greater.

TPA-induced EBV activation test Human B-lymphoblastoid Raji cells were incubated in 1 ml of RPMI 1640 medium containing sodium *n*-butyrate (3 m*M*), TPA (50 n*M*), and the test compound at 37°C under a 5% CO_2 atmosphere for 48 h. EBV activation was estimated by detection of EA using the indirect immunofluorescence method.[21] Smears were made from a cell suspension, and stained with high-titer EA-positive sera from anaplastic NPC patients followed by FITC-labeled anti-IgG. The percentage of EA-induced cells was compared to that of a control experiment with only sodium *n*-butyrate and TPA, in which the percentage of EA-induced cells was ordinarily around 50%. Cell viability was measured by the trypan blue-exclusion test. Every test was done in duplicate, and the mean value obtained.

Two-stage carcinogenesis experiment The anti-tumor-promoting activity of auraptene was examined by a standard initiation-promotion protocol with DMBA and TPA as previously reported.[28] One group was composed of 15 female ICR mice housed 5 per cage. The mice were given commercial rodent pellets (CE-2, Clea Japan, Inc., Tokyo) and fresh tap water *ad libitum*, both of which were provided freshly twice a week. The back of each mouse was shaved with a surgical clipper two days before initiation. The mice at 7 weeks old were initiated with topical application of DMBA (0.19 μmol/0.1 ml in acetone). One week after initiation, the mice were promoted with topical application of TPA (1.6 nmol/0.1 ml in acetone) twice a week for 20 weeks. In two other groups, the mice were given topical application of auraptene (16 nmol or 160 nmol/0.1 ml in acetone) 40 min before each TPA treatment. Anti-tumor-promoting activity was eval-

uated by analyzing the percentage of mice developing tumors and the number of tumors, more than 1 mm in diameter, per mouse. The data were statistically analyzed using Student's t test for the average number of tumors per mouse and the χ^2-test for the percentage of tumor-bearing mice.

TPA-induced superoxide generation test The test for inhibition of TPA-induced superoxide generation was done as previously reported.[28] Human promyelocytic leukemia HL-60 cells were inoculated at 5×10^5 cells/ml in RPMI 1640 supplemented with 10% FBS. The cells were preincubated with 1.25% dimethyl sulfoxide (DMSO) at 37°C in a 5% CO_2 incubator for 4 days to stimulate their differentiation into granulocyte-like cells. The cells were washed with phosphate-buffered saline (PBS), and suspended at a density of 1×10^6 cells/ml. The test compound, dissolved in 5 μl of DMSO, was added to the cell suspension, and the mixture thus obtained was incubated at 37°C for 15 min. The cells were washed with PBS twice to remove extracellular test compounds. Ninety seconds after stimulation with 5 μl of TPA solution (20 μM), 50 μl of cytochrome c solution (20 mg/ml) was added to the reaction mixture, which was incubated for another 15 min. The reaction was stopped by the addition of 5 μl of superoxide dismutase solution (15,000 units/ml). The reaction mixture was centrifuged at 2000g for 30 s, and the visible absorption of the supernatant at 550 nm was measured. The level of O_2^- production was calculated by use of the following equation[36]:

$$O_2^-(\text{nmol/ml}) = 47.7\times A_{550nm}$$

Every test was done in duplicate, and the mean value obtained.

Hydroperoxide formation test HL-60 cells were preincubated with 1.25% DMSO at 37°C in a 5% CO_2 incubator for 4 days to stimulate their differentiation into granulocyte-like cells. After having been washed with PBS twice, the cells were suspended at a density of 1×10^6 cells/ml. Hydroperoxides were detected by using DCFH-DA as an intracellular fluorescence probe.[37] Five microliters of DCFH-DA solution (200 μM) was added to the cell suspension, and the cells were incubated at 37°C for 15 min. After addition of the test compound dissolved in 5 μl of DMSO to the cell suspension, the mixture was incubated at 37°C for 15 min, and then 10 μl of a TPA solution (10 μM) was added. After 15 min, the reaction was stopped by adding 50 μl of an EDTA solution (800 μM). The cells were washed with PBS, then 2′,7′-dichlorofluorescein (DCF), formed by the reaction of DCFH with intracellular hydroperoxides, was detected by the use of a flow cytometer (CytoACE 150, JASCO, Tokyo). The TPA-treated cells showing fluorescence levels which were equal to or greater than the mean of the control cells plus 3 standard deviations were regarded as hydroperoxide positive (HP), and their percentage was expressed as hydroperoxide-positive percentage (HPP).

Quantitative analysis of auraptene Satsuma mandarin (*Citrus unshiu*), *natsumikan* (*C. natsudaidai*), *hassaku* orange (*C. hassaku*), Valencia orange (*C. sinensis*), navel orange (*C. brasiliensis*), lemon (*C. lemon*), lime (*C. aurantifolia*), Marsh and Star ruby grapefruit (white and ruby colors in sarcocarps, respectively, *C. paradisi*), and 9 brands of bottled fruit juices (compositions of fruits are listed in Table II) were purchased at Japanese markets in 1995. Fifty milliliters of a bottled juice or homogenized fresh fruit, separated into sarcocarp and peel, was extracted with chloroform at room temperature, and the organic solution was concentrated *in vacuo*. The dried samples thus obtained were dissolved in 1 ml of ethyl acetate, and subjected to HPLC analysis (column, Waters μBondasphere C_{18}, 3.9×150 mm; elute, 75% methanol in water; flow rate, 1.0 ml/min; detection, UV_{320nm}). Under these conditions, auraptene was detected at a retention time of 18.0 min. The peak area corresponding to auraptene was calculated by a Chromatopac C-R6A (Shimadzu, Kyoto) to determine the quantity.

RESULTS

The active constituents of *natsumikan* Whole fresh *natsumikan* were extracted by an FMC In-line Citrus Juice Extractor to give cold-pressed oil. The oil was then fractionated by silica gel column chromatography. The EBV activation inhibitors were finally purified by using HPLC to give auraptene (7-geranyloxycoumarin)[34] and umbelliferone (7-hydroxycoumarin)[35] (Fig. 1). Purified auraptene and umbelliferone were tested for inhibition of EBV activation. A chemopreventive phytochemical, genistein, known to be a tyrosine kinase inhibitor and also an antioxidant,[38-42] was used as a positive control. As shown in Fig. 2, auraptene and genistein at a concentration of 100 μM exhibited strong inhibitory activity toward EBV activation (inhibitory effect: IE=89% and

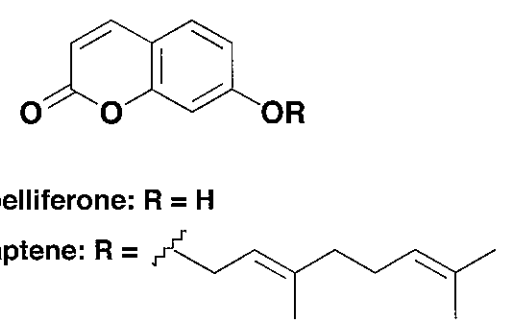

Fig. 1. Chemical structures of umbelliferone and auraptene.

83%, respectively), while umbelliferone showed weak inhibition (IE=20%).

Anti-tumor-promoting activity of auraptene As mentioned above, many inhibitors of EBV activation were found to have anti-tumor-promoting activity in mouse skin.[16, 21, 23, 24] Anti-tumor-promoting activity of auraptene was thus examined in a two-stage carcinogenesis experiment in mouse skin. As shown in Fig. 3, tumors

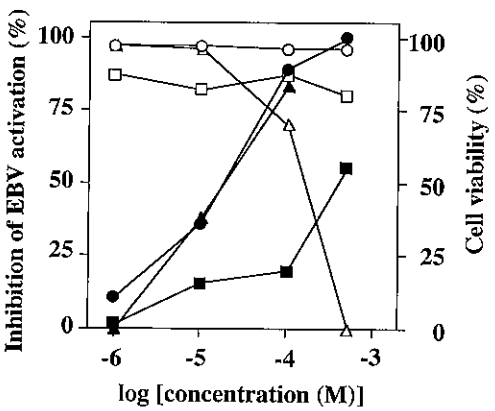

Fig. 2. Concentration-dependent inhibition of EBV activation by auraptene, umbelliferone, and genistein. Human B-lymphoblastoid Raji cells latently infected with EBV were incubated with *n*-butyrate (3 m*M*), TPA (50 n*M*), and auraptene (●, ○), umbelliferone (■, □), or genistein (▲, △) at 37°C for 48 h. The EBV-early antigen (EA) was detected by the indirect immunofluorescence method. The solid and open symbols represent inhibitory activity and cell viability, respectively.

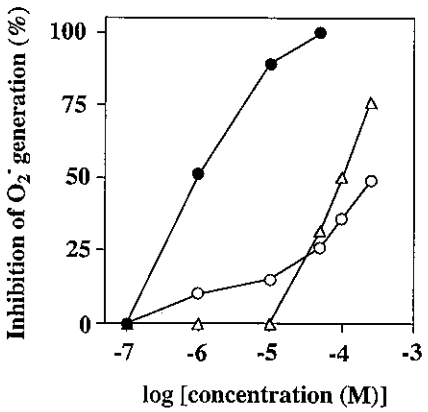

Fig. 4. Inhibitory effects of auraptene, umbelliferone, and genistein on superoxide generation in differentiated HL-60 cells. HL-60 cells were preincubated with 1.25% DMSO at 37°C for 4 days, differentiating them into granulocyte-like cells. Auraptene (●), umbelliferone (○), or genistein (△) solution was added to the cell suspension, and the mixture was incubated at 37°C for 15 min. Ninety seconds after stimulation with TPA (100 n*M*), cytochrome *c* solution was added to the reaction mixture. After 15 min the reaction mixture was centrifuged, and visible absorption at 550 nm was measured.

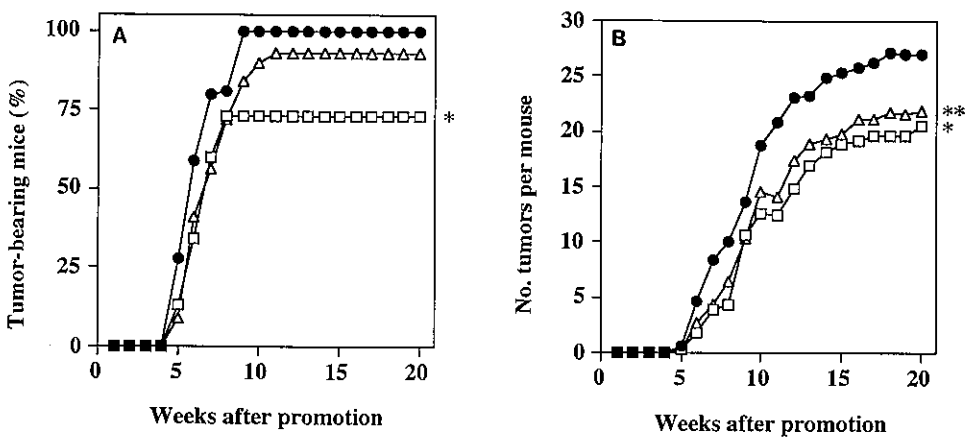

Fig. 3. Anti-tumor-promoting activity of auraptene in ICR mouse skin. Each group was composed of 15 female ICR mice. Mice at 7 weeks old were initiated with DMBA (0.19 μmol). One week after initiation, the mice were promoted with TPA (1.6 nmol, ●) twice a week for 20 weeks. In the inhibitor-treated groups, the mice were treated with 16 (△) or 160 (□) nmol of auraptene at 40 min prior to each TPA treatment. The anti-tumor-promoting activity was evaluated in terms of both the percentage of tumor-bearing mice (A) and the number of tumors per mouse (B). Statistical analysis was done by use of the χ^2-test on the percentage of tumor-bearing mice and Student's *t* test on the number of tumors per mouse. * $P<0.01$, ** $P<0.05$.

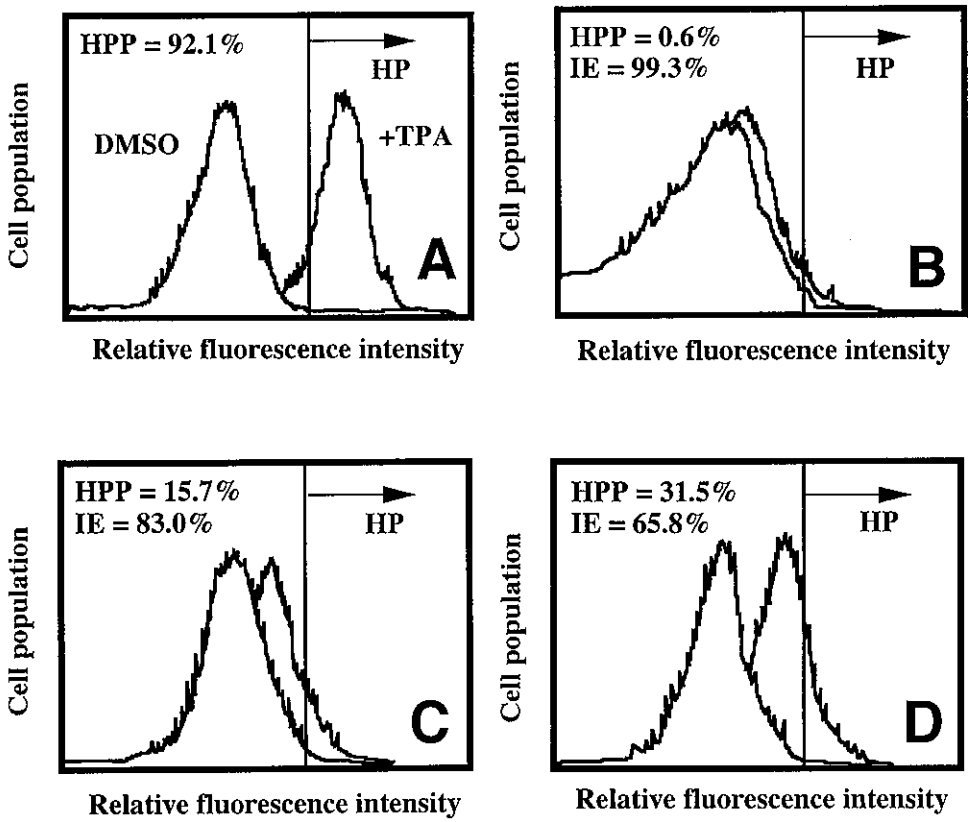

Fig. 5. Suppression of intracellular hydroperoxide formation by auraptene in differentiated HL-60 cells. The TPA-treated cells showing fluorescence levels which are equal to or greater than the mean control value plus 3 standard deviations were regarded as hydroperoxide positive (HP), and their percentage was expressed as the hydroperoxide-positive percentage (HPP). A, TPA (100 n*M*) alone; B, 100 μM auraptene+TPA (100 n*M*); C, 10 μM auraptene+TPA (100 n*M*); D, 5 μM auraptene+TPA (100 n*M*). IE: inhibitory effect (%).

began to appear at 5 weeks after continued tumor-promoting treatment in each group. The percentage of tumor-bearing mice and the average number of tumors per mouse in the control group reached 100% and 26.9, respectively, at the final week (20 weeks) of the experiment. In the group treated with 160 nmol of auraptene 40 min prior to each TPA treatment, the average number of tumors per mouse and the percentage of tumor-bearing mice were reduced by 23% ($P<0.05$ in t test) and by 27% ($P<0.01$ in χ^2-test), respectively. Auraptene even at 16 nmol significantly reduced the average number of tumors per mouse by 19% ($P<0.01$ in t test).

Suppressive effect of auraptene on superoxide (O_2^-) generation Oxidative stress is one of the critical biological responses induced by tumor promoters.[43] In particular, TPA-type tumor promoters are reported to trigger superoxide (O_2^-) generation in epithelial cells and leukocytes through the xanthine/xanthine oxidase (XA/XOD)[44] and NADPH oxidase systems,[45] respectively. Auraptene and umbelliferone neither inhibited XOD activity nor scavenged O_2^- up to a concentration of 100 μM in the XA/XOD system (data not shown). Some coumarin derivatives have recently been reported to inhibit O_2^- generation by activated polymorphonuclear leukocytes.[46] Therefore, auraptene was examined for the ability to inhibit O_2^- generation by HL-60 cells. Fig. 4 shows the concentration-dependence curves of auraptene, umbelliferone, and genistein for suppressive activity toward O_2^- generation. The O_2^- concentration in the control group treated only with 100 n*M* TPA was 18 nmol/ml, 15 min after TPA stimulation. Auraptene at a concentration of 10 μM inhibited O_2^- generation by 89% ($IC_{50}=1.2\ \mu M$), while umbelliferone ($IC_{50}=290\ \mu M$) and genistein ($IC_{50}=102\ \mu M$) showed little inhibitory activity.

Suppressive effect of auraptene on hydroperoxide formation The inhibitory activity of auraptene toward O_2^- generation in differentiated HL-60 cells led us to address the inhibitory efficacy against hydroperoxide (ROOH)

Table I. The Minimum Contents of Auraptene in Peel and Sarcocarp of Various Fruits

Fruit	Peel (mg/kg fresh wt.)	Sarcocarp (mg/kg fresh wt.)
Satsuma mandarin (*Citrus unshiu*)	0.6	<0.1
Natsumikan (*C. natsudaidai*)	407.8	837.9
Hassaku orange (*C. hassaku*)	585.2	331.1
Valencia orange (*C. sinensis*)	<0.1	<0.1
Navel orange (*C. brasiliensis*)	<0.1	<0.1
Lemon (*C. lemon*)	<0.1	12.8
Lime (*C. aurantifolia*)	<0.1	<0.1
Marsh grapefruit (white color, *C. paradisi*)	101.0	2,479.8
Star ruby grapefruit (ruby color, *C. paradisi*)	120.0	568.6

Table II. The Minimum Contents of Auraptene in Bottled Fresh Fruit Juices

Brand No.	Type of juice	Fresh juice content (%)	Auraptene content (mg/liter)
1	*Hassaku* orange, *natsumikan*	100	1.10
2	*Hassaku* orange	100	0.89
3	*Hassaku* orange	50	1.31
4	*Hassaku* orange, *natsumikan*, *iyokan*	10	0.10
5	*Hassaku* orange, *natsumikan*	10	0.26
6	Grapefruit	100	1.80
7	Valencia orange	100	<0.01
8	Valencia orange	100	0.02
9	Satauma mandarin (*Unshiu mikan*)	100	0.14

formation using DCFH-DA as an intracellular fluorescence probe.[37] Fig. 5 shows the cytograms of differentiated HL-60 cells treated with or without 100 n*M* TPA. HPP indicates the percentage of TPA-pretreated cells exhibiting fluorescence levels equal to or greater than the mean of those in control cells plus 3 standard deviations. As shown in panel A, most of the cells produced ROOHs (HPP=92.1%) with 100 n*M* TPA stimulation. Auraptene at a concentration of 100 μM (HPP=0.6%, IE=99.3%: panel B) or 10 μM (HPP=15.7%, IE=83.0%: panel C) markedly inhibited ROOH formation by 100 n*M* TPA, and significantly suppressed it even at 5 μM (HPP=31.5%, IE=65.8%, panel D).

The quantitative analysis of auraptene The results of quantitative analyses of auraptene in 9 species of fresh fruits and 9 brands of commercially available bottled fruit juices are shown in Tables I and II, respectively. As shown in Table I, high (>400 mg/kg fresh wt.) contents of auraptene were observed in the peels of *natsumikan* (*C. natsudaidai*) and *hassaku* orange (*C. hassaku*), and moderate (>100 mg/kg fresh wt.) contents in grapefruit (*C. paradisi*, white and ruby colors), while auraptene was not detected (<1 mg/kg fresh wt.) in the peels of Satsuma mandarin (*C. unshiu*), Valencia orange (*C. sinensis*), navel orange (*C. brasiliensis*), lemon (*C. lemon*), and lime (*C. aurantifolia*). Auraptene content in the sarcocarp of various fruits tended to be similar to that in the peel. However, the content in the sarcocarp of Marsh grapefruit (2,479.8 μg/kg fresh wt.) was remarkably high among the fruits analyzed. Table II shows the contents of auraptene in the bottled fruit juices. Juices from *natsumikan*, *hassaku* orange or grapefruit showed relatively higher contents of auraptene (brands No. 1–3, and 6: 0.89–1.80 mg/liter). However, even when *hassaku* orange or *natsumikan* was added to the juices, the auraptene content was trivial in the juices in which the total fruit juice content was 10% (brands 4 and 5; 0.10–0.26 mg/liter), compared with those (50–100%) in brands 1–3 (0.89–1.31 mg/liter).

DISCUSSION

The present study was conducted to search for new candidate chemopreventers from *natsumikan*. The IC_{50} value of auraptene ($IC_{50}=18$ μM) against EBV activation was equal to that of genistein ($IC_{50}=19$ μM), an isoflavonoid-type chemopreventer from soybean,[38–42] and lower than that of β-carotene ($IC_{50}=30$ μM, ref. 21). It is interesting to note that umbelliferone, lacking a geranyloxyl group present in auraptene, was shown to

have a much lower inhibitory activity (IC_{50}=450 μM). The importance of the geranyloxyl group for inhibitory activity may be related to a higher cellular uptake rate of auraptene or more favorable hydrophobic interactions with the target site(s). It is also notable that neither auraptene nor umbelliferone showed detectable cytotoxicity at a concentration of 500 μM, while genistein exhibited marked cytotoxicity at the same concentration (viability $<0.1\%$).

Auraptene has previously been reported to have spasmolytic activity,[47] activity to induce spontaneous beating of mouse myocardial cells,[48] inhibitory activity against aggregation and ATP release of rabbit platelets,[49] and anti-tumor activity against L1210 cells.[50] Coumarin-related compounds may exert anti-carcinogenic effects in rodents because of their modulation of phase II enzymes and their conjugation with electrophilic forms of carcinogenic metabolites.[51–57] In contrast, their modulating effects on tumor promotion are less well known, although inhibitory effects of coumarins on TPA-induced ^{32}Pi incorporation into phospholipids *in vitro* have been reported.[54] In addition, Nishino *et al.* reported anti-tumor-promoting activity of a coumarin-related mixture, Pd-II [(+) anomalin, (+) praeruptorin B], in mouse skin initiated with DMBA.[55] The present study supports their conclusion that coumarins are potential agents for anti-tumor promotion.

The mode of action by which auraptene inhibits tumor promotion has not yet been clarified. However, suppression of TPA-induced oxidative stress is presumably involved, since free radicals are relevant to tumor-promoting processes in the mouse skin model.[43] Following the application of TPA to mouse skin, leukocytes such as neutrophils, recruited by chemotactic factors, accumulate in the dermis and generate O_2^- through the NADPH oxidase system.[43] In this regard, it should be noted that the IC_{50} value of auraptene (1.2 μM) in the O_2^- generation inhibition assay was very much lower than those of umbelliferone (290 μM) and genistein (102 μM). Further, the IC_{50} value of auraptene is comparable to or lower than that of 1′-acetoxychavicol acetate (IC_{50}=4.3 μM), which we recently reported as a potent inhibitor of TPA-induced EBV activation,[20] O_2^- generation,[23] tumor promotion in mouse skin,[23] 4-NQO-induced rat tongue carcinogenesis,[25] and AOM-induced rat colonic ACF formation.[26] In the assay, after preincubation with test compounds, the cells were washed with PBS twice to remove extracellular test compounds. The O_2^- scavenging effects of residual test compounds, thus, should be negligible. It is well-known that O_2^- is converted to hydrogen peroxide (H_2O_2) either by superoxide dismutase or non-enzymatically in biological systems. The hydroxyl radical (OH·) formed from H_2O_2 randomly reacts with biological components within the cell. Recently Takeuchi *et al.* reported that OH· may directly induce the formation of 8-hydroxydeoxyguanosine in DMSO-differentiated HL-60 cells.[56] Alternatively, OH· may react with membrane lipids to form ROOHs, which are then converted to mutagenic, reactive carbonyl compounds such as malondialdehyde.[57]

NADPH oxidase is known to play a major role in O_2^- generation in phagocytes such as macrophages, neutrophils, or granulocytes.[45] The multicomponent NADPH oxidase system consists of heterodimeric cytochrome *b*, consisting of β-subunit (gp91-*phox*) and α-subunit (p22-*phox*) associated with p47-*phox* and p67-*phox*.[58] As mentioned above, since it has no O_2^--scavenging potential, auraptene may block the assembly or upstream signal transduction systems involved in the activation of the NADPH oxidase system.

The acetyl groups of DCFH-DA are hydrolyzed by esterase(s) to form DCFH, which is trapped within the cell. The reaction of DCFH with ROOHs generates a fluorescent compound, DCF, which is detectable by a flow cytometer.[37] Suppression of ROOH formation in differentiated HL-60 cells by auraptene (panels B–D in Fig. 5) might be attributable, at least in part, to the inhibition of O_2^- generation, since a part of ROOHs is considered to be indirectly formed by O_2^-, and effective concentrations of auraptene for the inhibition of O_2^- generation and ROOH formation are comparable to each other, i.e., auraptene at 10 μM inhibited O_2^- generation and ROOH formation by 89% and 83%, respectively (Figs. 4 and 5). Thus, suppression of TPA-induced oxidative stress seems to be involved in the action mechanism by which auraptene inhibits tumor promotion.

Although coumarins are generally known to be found in various foods,[59] the occurrence or content of auraptene in citrus fruits and their processed juices has not been reported. Quantitative analyses revealed that auraptene occurs in both fresh fruits and the bottled juices of *natsumikan*, *hassaku* orange or grapefruit at relatively high levels (Tables I and II). There is a strong correlation between the occurrence of auraptene in the sarcocarp and in the peel (Table I). The net content in the sarcocarp, however, was about 40 to 1,800 times less than in the peel. It is tempting to speculate that auraptene may have a relatively low toxicity since it occurs in edible sarcocarp, and citrus peel oils are used as food additives. In accordance with this, a collaborative study recently showed that auraptene suppressed the formation of AOM-induced rat colonic ACF formation in a dose-dependent manner, showed no prominent toxicity and did not reduce body or liver weight up to a dose of 500 ppm in the diet (T. Tanaka *et al.*, manuscript in preparation). In addition, auraptene was recently found to be an effective inhibitor of 4-NQO-induced rat oral carcinogenesis (T. Tanaka *et al.*, personal communications).

In conclusion, auraptene, a citrus coumarin, inhibited TPA-induced skin tumor promotion in mice. A possible action mechanism for the inhibition of tumor promotion is suppression of O_2^- generation in leukocytes. As the cold-pressed oils of citrus fruits are produced on an industrial scale throughout the world, auraptene is readily available. Further mechanistic studies as well as chemopreventive studies on auraptene in other animal models are in progress.

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