Disaccharide Esters Screened for Inhibition of Tumor Necrosis Factor-α Release Are New anti-cancer Agents

Sachiko Okabe,¹ Masami Suganuma,¹ Yukiko Tada,¹ Yumiko Ochiai,¹ Eisaburo Sueoka,¹ Hidehiko Kohya,² Akihiro Shibata,² Masami Takahashi,² Masashi Mizutani,³ Toshiake Matsuzaki⁴ and Hiroya Fujiki¹,⁵

¹Saitama Cancer Center Research Institute, Ina, Kitadai-gun, Saitama 362-0806. ²SSP Co., Ltd. Central Research Laboratories, 1143 Nanpei-dai, Narita 286-8511. ³Tobacco Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegawa, Aoba-ku, Yokohama 227-0052 and ⁴Leaf Tobacco Research Laboratory, Japan Tobacco Inc., Oyama, Tochigi 323-0808

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine playing a part in various pathological states. Non-toxic inhibitors of TNF-α release are thought to be promising agents for cancer prevention. We found that the acetone fraction of the tobacco leaf surface lipid containing glucose esters and sucrose esters inhibited both TNF-α release from BALB/3T3 and KATO III cells induced by okadaic acid and tumor promotion by okadaic acid on mouse skin initiated with 7,12-dimethylbenz(a)anthracene (DMBA). Next, we investigated the inhibition of TNF-α release with synthetic disaccharide esters, such as 6,6′-di-O-alkanoyl-α,α-trehaloses (6,6′-diester-trehaloses), 4,4′-di-O-alkanoyl-α,α-trehaloses (4,4′-diester-trehaloses) and 6,6′-diamino-6,6′-dideoxy-N,N′-dialkyanoyl-α,α-trehaloses (6,6′-diamide-trehaloses) bearing fatty acids of various chain lengths, and n-dodecyl-β-D-maltoside as a disaccharide monoester. 6,6′-Diester-trehaloses and 4,4′-diester-trehaloses of C₈ to C₁₂ fatty acids, 6,6′-diamide-trehaloses of C₄ to C₁₄ fatty acids, and n-dodecyl-β-D-maltoside all inhibited TNF-α release in a dose-dependent manner. The IC₅₀ values are 7.4–14.8 μM for 6,6′-diester-trehaloses (C₄ to C₁₂), 14.6–21.6 μM 4,4′-diester-trehaloses (C₄ to C₁₂), 2.9–15.0 μM for 6,6′-diamide-trehaloses (C₄ to C₁₄) and 23 μM for dodecyl-β-D-maltoside. Both 6,6′-di-O-octanoyl-α,α-trehalose (C₈, designated as SS555) and n-dodecyl-β-D-maltoside (C₁₂) inhibited tumor promotion by okadaic acid on mouse skin initiated with DMBA. Percentages of tumor-bearing mice in week 15 of tumor promotion were reduced from 60.0 to 13.3 with SS555, and to 46.7 with n-dodecyl-β-D-maltoside. Moreover, SS555 inhibited TNF-α gene expression mediated through inhibition of AP-1 activation, but not NF-κB activation. This paper reports that diester-trehaloses of C₄ to C₁₂ fatty acids and mimics of disaccharide monoesters such as n-dodecyl-β-D-maltoside appear to be potential cancer-preventive agents of a new type.

Key words: Tobacco leaf — Sucrose ester — Diester-trehalose — TNF-α — Inhibition of tumor promotion

In 1985, two diastereoisomers of 2,7,11-cembratriene-4,6-diol (α-CBT and β-CBT), isolated from cigarette smoke condensates and tobacco plants, were first reported to inhibit tumor promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA) on mouse skin initiated with 7,12-dimethylbenz(a)anthracene (DMBA). Based on the structural similarity of α-CBT to sarcophytol A, isolated from the soft coral sarcophyton glaucum, we found that sarcophytol A inhibited tumor promotion on mouse skin by three TPA-type tumor promoters (TPA itself, teleocidin, and aplysatoxin), and okadaic acid. Furthermore, sarcophytol A in the diet was effective in inhibiting chemical carcinogenesis in the colon, bladder and pancreas, and spontaneous tumor development in the liver, breast and thymus.

Our previous investigation revealed that inhibition of tumor necrosis factor-α (TNF-α) release, along with inhibition of TNF-α gene expression, is a new mode of cancer prevention. By assaying for inhibition of TNF-α release from cells treated with okadaic acid, such as mouse fibroblast cell line BALB/3T3 cells and human stomach cancer cell line KATO III cells, we screened for new potential anti-cancer agents from tobacco leaf extracts. Two fractions of the extracts, acetone fraction containing sugar esters and chloroform/acetone fraction containing α-CBT and β-CBT, showed inhibition of TNF-α release. Since the tobacco sugar esters are a mixture of glucose esters and sucrose esters of C₁₂ to C₁₄ fatty acids, the structure of the main sucrose ester was determined. Due to limitation of the availability of pure sugar esters, pure synthetic diester-trehaloses with fatty acids of various chain lengths were obtained. 6,6′-Di-O-alkanoyl-α,α-trehaloses (6,6′-diester-
trehaloses, 4,4′-di-O-alkanoyl-α,α-trehaloses (4,4′-diester-trehaloses) and 6,6′-diamino-6,6′-dideoxy-N,N′-dialkanoyl-α,α-trehaloses (6,6′-diamide-trehaloses) of fatty acids (C₃ to C₁₈) were used for further examination. We found that diester- and diamide-trehaloses required the optimum length fatty acid for inhibition of TNF-α release from cells. In addition, mimics of disaccharide monoester and glucose ester, such as n-dodecyl-β-D-maltoside (C₁₂) and n-octyl-β-D-glucopyranoside (C₈), were also studied. All the results showed that the disaccharide esters are active forms, while glucose ester is inactive. It was of interest to note that about 16 years ago a synthetic cord factor, 6,6′-di-O-decanoyl-α,α-trehalose (C₁₀, designated as SS554 as shown in Table II) was reported to have anti-tumor effects on transplanted fibrosarcoma in BALB/c mice by intratumoral injection.⁷ We show here that 6,6′-diester-trehaloses of fatty acids (C₈, C₁₀, and C₁₂) and n-dodecyl-β-D-maltoside are a new class of anti-cancer agents, and that their inhibitory effects on TNF-α gene expression and TNF-α release are mediated by inhibition of AP-1 activation.

MATERIALS AND METHODS

Materials 6,6′-Diester-trehaloses, 4,4′-diester-trehaloses and 6,6′-diamide-trehaloses of various fatty acids (C₃ to C₁₈) were synthesized as described previously,⁸ and n-octyl-β-D-glucopyranoside (C₈) and n-dodecyl-β-D-maltoside (C₁₂) were purchased from Wako Pure Chemical Inc., Ltd. (Osaka) (Fig. 1). Okadaic acid was isolated from a black sponge, Halicondria okadai, as described previously.⁹ DMBA was obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction and fractionation of tobacco leaf surface lipids Leaf surface lipids of tobacco plants (Nicotiana tabacum) were extracted by dipping fresh leaves in chloro-

Sugar esters in tobacco leaf

![Sugar esters in tobacco leaf, Glucose ester, Sucrose ester, Synthetic sugar esters, n-Octyl-β-D-glucopyranoside, n-Dodecyl-β-D-maltoside, 6,6′-Diester-trehalose, 4,4′-Diester-trehalose, 6,6′-Diamide-trehalose.](image)

Fig. 1. Structures of sugar esters from tobacco leaf, and synthetic sugar esters, such as n-octyl-β-D-glucopyranoside and n-dodecyl-β-D-maltoside, and 6,6′-diester-trehaloses, 4,4′-diester-trehaloses and 6,6′-diamide-trehaloses.
form twice for 10 s. After evaporation, extracts were applied to a silica gel (Wakoel C-200, Wako Pure Chemical Inc., Ltd.) column and eluted batch-wise with chloroform, chloroform/acetone (1/1), acetone, and methanol. The chloroform/acetone fraction contained α-CBT and β-CBT, and the acetone fraction contained glucose esters and sucrose esters. The structure of the main sucrose ester was determined by 1H-NMR and 13C-NMR analyses, mild alkaline hydrolysis, 2-D NMR using the HMBC (1H-detected multiple-bond heteronuclear multiple-quantum coherence) technique, and fast atom bombardment mass spectral analysis to be 6-O-acetyl-2,3,4-tri-O-acyl-α-d-glucopyranosyl-β-D-fructofuranoside. The structure was the same as that reported by Severson et al. in 1984.10)

Inhibition of TNF-α release from BALB/3T3 cells and KATO III cells
BALB/3T3 cells and KATO III cells were provided by the Japanese Cancer Research Resources Bank, Tokyo. BALB/3T3 cells were cultured in MEM medium and KATO III cells in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal bovine serum in a 5% CO2 atmosphere at 37 °C.10) The cells (2×10^5/well) were first incubated with the eluate at a concentration of 100 µM or various concentrations of synthetic compounds. One hour later, BALB/3T3 cells were treated with 200 nM okadaic acid to induce TNF-α release, and KATO III cells were treated with 50 nM okadaic acid, for another 24 h. The concentration of TNF-α in the medium was measured using an enzyme-linked immunosorbent assay (ELISA) kit for mouse TNF-α (Genzyme, Cambridge, MA) and human TNF-α (Amersham Pharmacia, Buckinghamshire, UK), as described previously.10) Okadaic acid-induced TNF-α release without pretreatment was expressed as 100%.

Inhibition of TNF-α gene expression in KATO III cells
KATO III cells (6×10^5) were first treated with 6,6'-diester-threo-taloses and, 1 h later, with 50 nM okadaic acid. After 14 h, total RNA was prepared according to the method of Chomczynski and Sacchi.11) The level of TNF-α mRNA was determined by the use of a GeneAmp RNA polymerase chain reaction (PCR) kit (Roche Molecular Systems, Inc., Branchburg, NJ). Total RNA (1 µg) was transcribed to cDNA by murine leukemia virus reverse transcriptase at 37°C for 1 h. One microliter of cDNA was amplified in 10 µl of GeneAmp PCR reaction mixture containing 100 nM 5' and 3' primers of TNF-α, 200 µM dNTP, 55 kBq of [32P]dCTP and 0.25 units of Ampli Taq Gold polymerase (Roche Molecular Systems, Inc.). PCR was performed in a DNA thermal cycler for 9 min at 95°C and for 32 cycles; 30 s denaturation at 94°C, 45 s annealing at 60°C, and 1 min extension at 72°C. 5' Primer and 3' primer for TNF-α and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized as reported previously.10) Radioactivity of TNF-α mRNA was measured by a BAS 2000 Image analyzer (Fuji Photo Film Co., Ltd., Tokyo) and normalized with respect to GAPDH mRNA as a quantitative control. TNF-α mRNA was expressed relative to that of the non-treated control.

Preparation of nuclear protein and electrophoretic mobility shift assay
Nuclear protein was prepared by the published method with some modifications.12) Briefly, the cells were treated in the same way as described for the experiment on TNF-α gene expression, and resuspended in 0.4 ml of cell lysis buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSE, 2 µg/ml leupeptin, 2 µg/ml aprotonin, 1 mM DTT and 0.6% NP-40. The mixture was centrifuged at 4°C for 30 s. The nuclei were then resuspended in 25 µl of nuclear protein extraction buffer containing 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSE, 2 µg/ml leupeptin, 2 µg/ml aprotonin, and 1 mM DTT. After centrifugation, the supernatant was used as the nuclear protein solution and stored at −80°C. AP-1 oligonucleotide 5'-CGCTTGTAGTGTCAGCGGAA-3' and NF-kB oligonucleotide 5'-AGTTAGGGGACTTTCCAGGCC-3' (Promega Corp., Madison, WI) were end-labeled with [32P]ATP. Nuclear extracts (4 µg) were added to the DNA binding buffer containing 0.1 mg/ml poly(dI-dC), 0.1 mg/ml BSA, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM MgCl2, 4% glycerol, 0.5 mM EDTA, and 0.5 mM DTT. The reaction mixture was incubated for 10 min and then further incubated for 30 min at room temperature after adding 3P-labeled oligonucleotide probe (3×10^4 cpm). The DNA-protein complexes were subjected to electrophoresis in 4% nondenaturing acrylamide gel and quantitation of the radioactive band was done with a BAS 2000 Image analyzer.

Inhibition of tumor promotion in CD-1 mouse skin
Female CD-1 mice were obtained from Charles River Japan Inc. (Kanagawa). Inhibition of tumor promotion in a two-stage carcinogenesis experiment on mouse skin was examined as described previously.13) Briefly, initiation was achieved by a single application of 100 µg of DMBA. Tumor promotion was conducted by repeated applications of okadaic acid (1.0 µg, 1.2 nmol per application), with each group consisting of 15 mice. The acetone fraction (No. 5) of tobacco leaf extracts (200 µg per application), 6,6'-di-O-octanoyl-α,α-trehalose (C9, SS55 as shown in Table II) (71.4 µg, 120 nmol per application) and n-dodecyl-β-D-maltoside (61.3 µg, 120 nmol per application) were separately applied 15 min before each application of okadaic acid. Inhibition of tumor promotion was estimated in terms of decrease in the percentage of tumor-bearing mice.

RESULTS

Inhibition of TNF-α release by tobacco leaf extracts
Flower and leaves of tobacco (Nicotiana tabacum) were
extracted with chloroform for 10 s two times, after which the concentrated extracts were subjected to silica gel column chromatography. Batch-wise elution resulted in four main fractions, chloroform, chloroform/acetone (1/1), acetone and methanol fractions (Table I). The chloroform/acetone fraction (No. 3 and 4), the acetone fraction (No. 5) and the methanol fraction (No. 6) at a concentration of 100 µg/ml strongly inhibited TNF-α release from BALB/3T3 cells, and KATO III cells treated with okadaic acid, whereas the chloroform fraction (No. 1) did not inhibit TNF-α release (Table I). The active fraction eluted with chloroform/acetone contained α-CBT and β-CBT as major components, which were identified by thin layer chromatography and 1H-NMR. However, the acetone fraction (No. 5) was found to contain sugar esters, specifically glucose esters and sucrose esters of C1 to C8 fatty acids (Fig. 1), but not α-CBT and β-CBT. The main compound of the sucrose esters was structurally determined to be 6-O-acetyl-2,3,4-tri-O-acyl-α-D-glucopyranosyl-β-D-fructofuranoside. Thus, we think that the sucrose esters contain new inhibitors of TNF-α release.

### Inhibition of TNF-α release with sugar esters

Since sugar esters in tobacco leaf extracts are a complex mixture of glucose esters and sucrose esters, we studied the inhibition of TNF-α release with synthetic disaccharide esters and monosaccharide ester. For synthetic disaccharide esters, 6,6′-diester-trehaloses, 4,4′-diester-trehaloses and 6,6′-diamide-trehaloses with fatty acids of various chain lengths from KATO III cells treated with okadaic acid. Pretreatment with 6,6′-diester-trehaloses for 1 h was followed by 50 nM okadaic acid. After 24 h incubation, the concentration of TNF-α in the medium was determined by using ELISA. Okadaic acid-induced TNF-α release (261 pg/ml) is expressed as 100%. 6,6′-Diester-trehaloses of C2 ( ), C4 ( ), C6 ( ), C8 ( ), C10 ( ) and C12 ( ) fatty acids.

### Table I. Inhibition of TNF-α Release by Tobacco Leaf Extracts

| Elution Fraction | Inhibition of TNF-α release (% of control) |
|------------------|------------------------------------------|
|                  | BALB/3T3 cells | KATO III cells |
| CHCl3            |               |                |
| 1                | 105.3±5.3     | 101.2±6.1      |
| 2                | 125.7±4.6     | 70.5±1.7       |
| CHCl3/acetone    |               |                |
| 3                | 42.4±30.7     | 28.5±6.4       |
| 4                | 31.8±4.5      | 25.3±0.4       |
| Acetone          |               |                |
| 5                | 23.9±7.9      | 24.9±6.0       |
| MeOH             |               |                |
| 6                | 0.0±0.0       | 29.3±1.6       |
| 7                | 78.8±0.1      | 81.3±0.7       |

### Table II. Inhibition of TNF-α Release from KATO III Cells by Synthetic Disaccharide Esters

| No. of carbon atom in fatty acid | Inhibition of TNF-α release (IC50 µM) |
|----------------------------------|--------------------------------------|
| C2                               | >100.0                               |
| C4                               | >100.0                               |
| C6                               | >100.0                               |
| C8                               | 14.8 (SS555)                         |
| C10                              | 10.0 (SS554)                         |
| C12                              | 7.4                                  |
| C14                              | >100.0                               |
| C16                              | >100.0                               |
| C18                              | n.d.                                 |

n.d.: not determined.

### Fig. 2. Inhibition of TNF-α release by 6,6′-diester-trehaloses with fatty acids of various chain lengths from KATO III cells treated with okadaic acid. Pretreatment with 6,6′-diester-trehaloses for 1 h was followed by 50 nM okadaic acid. After 24 h incubation, the concentration of TNF-α in the medium was determined by using ELISA. Okadaic acid-induced TNF-α release (261 pg/ml) is expressed as 100%. 6,6′-Diester-trehaloses of C2 ( ), C4 ( ), C6 ( ), C8 ( ), C10 ( ■ ) and C12 ( □ ) fatty acids.

### Fig. 3. Inhibition of TNF-α release by n-dodecyl-β-D-maltoside, but not by n-octyl-β-D-glucopyranoside or trehalose from KATO III cells treated with okadaic acid. n-Dodecyl-β-D-maltoside ( ● ), n-octyl-β-D-glucopyranoside ( ○ ) and trehalose ( △ ).
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lengths were first subjected to tests of inhibition of TNF-α release. Dose-response curves of 6,6′-diester-trehaloses with C_8 to C_{12} fatty acids in inhibition of TNF-α release from KATO III cells are shown in Fig. 2. 6,6′-Diester-trehaloses with C_8 to C_{12} fatty acids showed strong inhibitory activity, whereas short-chain-length (less than C_8) fatty acids showed reduced inhibitory activity (Fig. 2). 6,6′-Diester-trehaloses with longer chain length (more than C_{12}) fatty acids lost inhibitory activity due to poor solubility in the medium. Like 6,6′-diester-trehaloses, 4,4′-diester-trehaloses with C_8 to C_{12} fatty acids, and 6,6′-diamide-trehaloses with C_8 to C_{14} fatty acids showed inhibition of TNF-α release from KATO III cells (Table II). These results indicate that the optimal chain lengths of fatty acid of diester- and diamide-trehaloses for inhibition of TNF-α release are C_8 to C_{12} (Table II). As Fig. 2 and Table II show, the concentrations giving 50% inhibition (IC_{50}) were 14.8 µM for 6,6′-di-O-octanoyl-α,α-trehalose (SS555) and 10.0 µM for 6,6′-di-O-decanoyl-α,α-trehalose (SS554).

Next we studied the difference in inhibitory activity between disaccharide ester and monosaccharide ester using n-dodecyl-β-D-maltoside and n-octyl-β-D-glucopyranoside, based on our finding that the optimal chain length of fatty acids for diester-trehaloses is C_8 to C_{12}. Fig. 3 shows dose-response curves of n-dodecyl-β-D-maltoside, n-octyl-β-D-glucopyranoside and trehalose. n-Dodecyl-β-D-maltoside (C_{12}) inhibited TNF-α release (IC_{50}/23 µM) in the same manner as 6,6′-diester-trehaloses (C_8 to C_{12}), 4,4′-diester-trehaloses (C_8 to C_{12}), and 6,6′-diamide-trehaloses (C_8 to C_{14}) (Table II). However, n-octyl-β-D-glucopyranoside (C_8) and trehalose at concentrations up to 100 µM were ineffective (Fig. 3). The results indicated that disaccharides with one or more hydrophobic groups are active compounds, but the corresponding derivative of a monosaccharide is not. In addition, C_{10} to C_{14} fatty acids at concentrations up to 100 µM did not show any inhibitory activity (Table II).

Inhibition of TNF-α gene expression by SS555 mediated through inhibition of AP-1 binding activity. Okadaic acid increased TNF-α gene expression about 10–16 times in KATO III cells 14 h after treatment, whereas without okadaic acid, expression was not induced. Next, we examined whether SS555 (one of the diester-trehaloses) inhibits TNF-α gene expression in the cells. SS555 at concentrations of 1 to 100 µM dose-dependently inhibited the TNF-α mRNA level (Fig. 4A). 100 µM SS555 reduced it almost to the basal level. On the other hands, trehalose and 6,6′-di-O-acetyl-α,α-trehalose (C_3) at a concentration of 500 µM had no effect, but 500 µM 6,6′-di-O-caproyl-α,α-trehalose (C_3) weakly inhibited TNF-α at the mRNA level (Fig. 4B). These results were consistent with their potency for inhibition of TNF-α release, suggesting that diester-trehaloses inhibited TNF-α expression induced by okadaic acid at the transcriptional level. TNF-α gene expression is induced by activation of transcription factors, such as AP-1 and NF-κB. 13) Treatment of KATO III cells with okadaic acid strongly stimulated the DNA binding activity of AP-1 and NF-κB in nuclear extracts. Pretreatment with 100 µM SS555 inhibited AP-1 DNA binding activity about 70% (Fig. 5A), but had no effect on NF-κB DNA binding activity (Fig. 5B). These results suggested that inhibition of TNF-α gene expression by SS555 was partly mediated through inhibition of AP-1 activation.

Inhibition of tumor promotion by tobacco leaf extract (No. 5). SS555 and n-dodecyl-β-D-maltoside. Tumor promotion in the control group treated with DMBA plus okadaic acid resulted in 60.0% tumor-bearing mice and 1.5 average number of tumors/mouse in week 15 of tumor promotion. Treatment with tobacco leaf extract (No. 5) delayed development of the first tumor by four weeks compared with the control group, while reducing the per-

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**Fig. 4.** Inhibitory effects of 6,6′-diester-trehaloses with C_2, C_8 and C_6 fatty acids on TNF-α gene expression in KATO III cells. A: KATO III cells (3 × 10⁶) were first treated with various concentrations of 6,6′-di-O-octanoyl-α,α-trehalose (C_8, SS555) 1 h prior to 50 nM okadaic acid. A: SS555. B: Trehalose and 6,6′-diester-trehaloses with C_8 and C_6 fatty acids.

**Fig. 5.** DNA binding activity of AP-1 and NF-κB in nuclear extracts. Pretreatment with 100 µM SS555 inhibited AP-1 DNA binding activity about 70% (Fig. 5A), but had no effect on NF-κB DNA binding activity (Fig. 5B). These results suggested that inhibition of TNF-α gene expression by SS555 was partly mediated through inhibition of AP-1 activation.
The percentage of tumor-bearing mice from 60.0% to 40.0% (Fig. 6A) and the average number of tumors/mouse from 1.5 to 0.9 in week 15. Treatment with SS555 also delayed development of the first tumor by seven weeks, and the relevant numbers were reduced to 13.3% (Fig. 6B) and 0.1 in week 15. The inhibitory effect of SS555 was statistically signifi-
DISCUSSION

First, α-CBT and β-CBT were isolated from tobacco plants. They were screened for inhibition of the induction of Epstein-Barr virus early antigen in Raji cells by TPA.12 Sarcophytol A, derived from marine organisms, was screened since it is structurally related to α-CBT and β-CBT.22 Recently we confirmed that TNF-α is strongly involved in tumor promotion and progression: therefore, inhibition of TNF-α gene expression and TNF-α release constitutes a new target for cancer prevention.4, 5 Tobacco plants were re-examined as a potential new source of cancer-preventive agents using an assay of inhibition of TNF-α release from cells. The study revealed that disaccharide esters, including sucrose esters, diester-trehaloses and n-dodecyl-β-D-maltoside, are new inhibitors of TNF-α release, in addition to α-CBT and β-CBT. Thus, inhibition of TNF-α release from cells induced by okadaic acid is a new criterion in screening for cancer-preventive agents.

Tobacco sugar esters are a mixture of glucose esters and sucrose esters. One of the authors (T. M.) reported that sucrose esters of C6 to C12 fatty acids are inhibitors of tobacco seed germination and growth.21 Although the relationship between inhibition of tobacco seed germination and cancer prevention is a relatively new area for investigation, we studied the inhibition of TNF-α release by diester-trehaloses with fatty acids of various chain lengths and n-dodecyl-β-D-maltoside as examples of disaccharide di- and monooesters, and n-octyl-β-D-glucopyranoside as an example of a glucose ester. The results clearly showed that disaccharide esters inhibited TNF-α release from KATO III cells, whereas the glucose ester, n-octyl-β-D-glucopyranoside did not. Moreover, the fraction (No. 5) containing tobacco sugar esters, SS555 and n-dodecyl-β-D-maltoside, inhibited tumor promotion on mouse skin, indicating that disaccharide esters are a new class of inhibitors of tumor promotion. We previously reported that various compounds, such as sarcophytol A, canventol, (−)-epigallocatechin gallate (EGCG), tamoxifen, cryptoporic acid E, 1α, 25-(OH)2 vitamin D3 and quercetin, inhibited TNF-α release, and this new evidence suggests that inhibition of TNF-α is a common process of cancer inhibition.4, 5

Expression of TNF-α gene is regulated by various transcription factors, such as AP-1 and NF-κB; there are AP-1 and NF-κB binding sites in the promoter region of TNF-α gene.13, 14 As shown in Figs. 4 and 5, diester-trehaloses required the optimum length of fatty acids for their inhibition of TNF-α gene expression, and AP-1 binding activity was inhibited by treatment with SS555, suggesting that diester-trehaloses with C6 to C12 fatty acid inhibit TNF-α transcription partly through inhibition of AP-1 activation. Inhibition of AP-1 activation is mediated through inhibition of the c-Jun NH2-protein kinase dependent pathway, and it has been reported that various compounds, such as EGCG and theaflavins, inhibited AP-1 activation and tumor promotion.15 Caffeic acid phenethyl ester (CAPE), an active compound isolated from the propolis of honeybee hives, and curcumin are inhibitors of phorbol ester-induced H2O2 production and tumor promotion.12, 16 Recently it was also reported that CAPE and curcumin completely blocked the activation of NFκB by TNF-α. All these findings, considered together, indicate that reduction of TNF-α expression in cancerous tissue leads to inhibition of cancer development, and probably to prevention of cancer. Among various inhibitors so far discovered, disaccharide esters including sucrose esters, diester-trehaloses and n-dodecyl-β-D-maltoside are a new class of inhibitors of cancer development.

Inhibition of TNF-α expression is associated with inhibition of interleukin (IL)-1, IL-6, and IL-10 expression.17 Administration of SS555 into mouse stomach inhibited tumor development in subcutaneous tissue associated with a reduction of serum concentrations of TNF-α, IL-1 and IL-6, suggesting that SS555 also inhibits cachexia in tumor-bearing mice (data not shown). Since SS555 is nontoxic to mice, it might represent a new lead compound for cancer preventive agents.

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REFERENCES

1) Saito, Y., Takizawa, H., Konishi, S., Yoshida, D. and Mizusaki, S. Identification of cembratriene-4,6-diol as anti-tumor-promoting agent from cigarette smoke condensate. *Carcinogenesis*, 6, 1189–1194 (1985).

2) Kobayashi, M., Nakagawa, T. and Mitsuhashi, H. Marine terpenes and terpenoids. I. Structures of four cembrane-type diterpenes; sarcophytol-A, sarcophytol-A acetate, sarcophytol-B, and sarcophytin-A, from the soft coral, *Sarcophytum glaucum*. Chem. Pharm. Bull. (Tokyo), 27, 2382–2387 (1979).

3) Fujiki, H., Suganuma, M., Yoshizawa, S., Yatsunami, J., Nishiwaki, S., Furuya, H., Okabe, S., Nishiwaki-Matsushima, R., Matsunaga, S., Muto, Y., Okuda, T. and Sugimura, T. Sarcophytol A and (−)-epigallocatechin gallate (EGCG), nontoxic inhibitors of cancer development. *In Cancer Chemoprevention*, ed. L. Wattenberg, M. Lipkin, C. W. Boone and G. F. Kelloff, pp. 393–403 (1992). CRC Press Inc., Boca Raton, FL.

4) Fujiki, H., Komori, A. and Suganuma, M. Chemoprevention of cancer. *In Comprehensive Toxicology,* ed. G. T. Bowden and S. M. Fischer, vol. 12, pp. 453–471 (1997). Pergamon, Cambridge, UK.

5) Suganuma, M., Okabe, S., Sueoka, E., Iida, N., Komori, A., Kim, S.-J. and Fujiki, H. A new process of cancer prevention mediated through inhibition of tumor necrosis factor-α expression. *Cancer Res.*, 56, 3711–3715 (1996).

6) Matsuizaki, T., Koseki, K. and Koizumi, A. Germination and growth inhibition of surface lipids from *Nicotiana* species and identification of sucrose esters. *Agric. Biol. Chem.*, 52, 1889–1897 (1988).

7) Kohya, H., Ishii, F., Takano, S., Katori, T., Ebina, T. and Ishida, N. Antitumor effect of a synthetic cord factor, 6,6′-di-O-decanoyl-α,α-trehalose (SS554), in mice. *Jpn. J. Cancer Res. (Gann)*, 77, 602–609 (1986).

8) Nishikawa, Y., Yoshimoto, K., Okada, M., Ikekawa, T., Abiko, N. and Fukuoka, F. Chemical and biochemical studies on carbohydrate esters. V. Anti-Ehrlich ascites tumor effect and chromatographic behavior of fatty acid monoesters of sucrose and trehalose. *Chem. Pharm. Bull. (Tokyo)*, 25, 1717–1724 (1977).

9) Severson, R. F., Arrendale, R. F., Chortyk, O. T., Johnson, A. W., Jackson, D. M., Gwynn, G. R., Chaplin, J. F. and Stephenson, M. G. Quantitation of the major cuticular components from green leaf of different tobacco types. *J. Agric. Food Chem.*, 32, 566–570 (1984).

10) Sueoka, N., Sueoka, E., Okabe, S. and Fujiki, H. Anti-cancer effects of morphine through inhibition of tumour necrosis factor-α release and mRNA expression. *Carcinogenesis*, 17, 2337–2341 (1996).

11) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, 162, 156–159 (1987).

12) Natarajan, K., Singh, S., Burke, T. R., Jr., Grunberger, D. and Aggarwal, B. B. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-κB. *Proc. Natl. Acad. Sci. USA*, 93, 9090–9095 (1996).

13) Spriggs, D. R., Deutsch, S. and Kufe, D. W. Genomic structure, induction, and production of TNF-α. *In Tumor Necrosis Factors*, ed. B. B. Aggarwal and J. Vilcek, pp. 3–34 (1992). Marcel Dekker, Inc., New York.

14) Aggarwal, B. B. and Natarajan, K. Tumor necrosis factors: developments during the last decade. *Eur. Cytokine Netw.*, 7, 93–124 (1996).

15) Dong, Z., Ma, W.-Y., Huang, C. and Yang, C. S. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (−)-epigallocatechin gallate, and theaflavins. *Cancer Res.*, 57, 4414–4419 (1997).

16) Singh, S. and Aggarwal, B. B. Activation of transcription factor NF-κB is suppressed by curcumin (diferulolylmethane). *J. Biol. Chem.*, 270, 24995–25000 (1995).

17) Neta, R., Sayers, T. J. and Oppenheim, J. J. Relationship of TNF to interleukins. *In “Tumor Necrosis Factors,”* ed. B. B. Aggarwal and J. Vilcek, pp. 499–566 (1992). Marcel Dekker, Inc., New York.