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Synthesis and Cytotoxic Effects of Deoxy-tomentellin

Du Seok Han, Kui Ho Jung, Woo Young Jung, In Kyo Oh[1], Kil Ung Kang[2], and Seung Hwa Baek[2]

Department of Oral Anatomy, School of Dentistry, and [2]Department of Natural Products, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea, [1]Department of Environmental Industry, Wonkwang Health College, Iksan 570-750, Korea.

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Cannabigerol (**1**, CBG), methyl 4-[(2*E*)-3,7-dimethyl-2,6-octadienyl)oxy]-3-methoxybenzoate (**2**, DTM), 5-fluorouracil (**3**, FU) as a reference, and cannabidiol (**4**, CBD) were tested for their growth inhibitory effects against KB(ATCC NO, OCL 17) cell lines using two different assays, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT) assay and the sulforhod-amine B protein (SRB) assay. These compounds showed inhibitory activity *in vitro* in the micromolar range against KB cell lines. In general, the antitumor activities of these compounds (**1**, **2**, **3** and **4**) were dose-dependent over the micromolar concentration range of 1 to 100 M. The comparison of IC_{50} values of these compounds in tumor cell lines showed that their susceptibility to these compounds decreases in the following order: DTM > CBD > 5-FU > CBG by MTT assay and DTM = CBD > 5-FU > CBG by SRB assay. CBG **1**, DTM **2**, 5-FU **3**, and CBD **4** were tested for their cytotoxic effects on NIH 3T3 fibroblasts using two different assays, the MTT assay and SRB assay. These compounds exhibited potent cytotoxic activities *in vitro* in the micromolar range against NIH 3T3 fibroblasts. In general, the cytotoxic acivities of these compounds (**1**, **2**, **3** and **4**) were dose-dependent over the micromolar concentraion range of 1 to 100 M. The comparison of CD_{50} values of these compounds in NIH 3T3 fibroblasts shows that their susceptibility to these compounds in decreases the following order(:) CBD > 5-FU > DTM > CBG by MTT assay, CBD > 5-FU > CBG > DTM by SRB assay. These results suggest that DTM 2 has the most growth-inhibitory activity against KB cell lines.

Key words: Cannabigerol, Methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyl)oxy]-3-methoxy benzoate, 5-Fluorouracil, Cannabidiol

INTRODUCTION

Liverworts have yielded many new compounds, mainly terpenoids or lipophilic aromatic compounds, a good proportion of which were found to be active (Asakawa, 1995). The cytotoxic activity of crude extracts of *Trichocolea mollissima* from New Zealand, a species whose chemistry had been reported (Perry *et al.*, 1996), sparked renewed interest in liverwort *Trichocolea*. This foliose liverwort grows throughout New Zealand in the rain forest and beach forest, hanging from old trunks or growing erect on the forest floor (Allison *et al.*, 1975). Asakawa *et al.* (1981) have suggested that the isoprenyl benzoates which they reported could serve as significant chemisystemic markers of *T. tomentella* and liverworts of the *Trichocolaceae* (Asakawa *et al.*, 1981). After revising the structure of *Tomentellin*, the characteristic compounds of the *Trichocolaceae* seems to be the 4-isoprenyl ethers of methyl vanillate. A chemotaxonomic pattern can be established from the extractions of common New Zealand *Trichocolaceae, T. lanata, T. mollissima,* and *T. hatcheri,* based on the presence of the 4-isoprenyl ethers. These discoveries prompted us to investigate another New Zealand's *Trichocolea* species, *T. hatcheri Hodgs*. This species, which grows throughout New Zealand, can be distinguished from *T. mollissima* by its smaller size, dark green color and prostrate habit (Perry *et al.*, 1996). We reported on that DTM **2** from *T. hatcheri.* showed no cytotoxic effects against monkey kidney (BSC) cells and antifungal activity against the dermatophyte *Trichophyton mintagrophytes*.

In the present study, we investigated inhibitory effects

Correspondence to: Seung Hwa Baek, Department of Natural Products, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea.
E-mail: shbaek@wonkwang.ac.kr

of DTM against KB cell lines using two different assays, the MTTand SRB assays. The effects of 5-FU were also examined for comparison.

MATERIALS AND METHODS

Melting points were determined on a Kofler hot stage and were uncorrected. ^{1}H and ^{13}C-NMR, HMQC, HMBC, and NOE spectra were recorded using Varian Gemini-200 and Varian VXR-300 spectrometers. When $CDCl_3$ was used as a solvent, $CHCl_3$ (1H, δH, 7.27) or $CDCl_3$ (^{13}C, δC, 77.08) was used as an internal reference. Elemental analyses were performed by microanalytical laboratories, Otago University. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrophotometer. Mass spectra were recorded on a Varian Mat CH-5 mass spectrometer. TLC was carried out on Si gel 60 F_{254} precoated 0.2 mm aluminium sheet (Merck 5562). Developed plates were visualized by UV light and staining with a 5% solution of anisaldehyde in ethanol. Flash chromatography was carried out with a Silica gel 230-400 mesh.

Medium pressure liquid chromatography was performed on an a 1 meter long ALTEX glass column having an internal diameter of 9mm using an FMI pump, and silica gel 60 (230-400 mesh) purchased from Merck. Fractions were collected with LKB 2070 or LKB 7000 fraction collectors at a rate of 2-10 ml/min.

Materials

5-FU, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazoliumbromide, fetal bovine serum (FBS), sulforhodamine B protein, streptomycin, and penicillin were obtained from Sigma Chemical Co., Ltd. (St. Louis, USA). Geranyl bromide, sodium hydride and ethyl iodide were purchased from Aldrich Chemical Co., Ltd. (Milwaukee, U.S.A.). 3-Methyl-4-hydroxybenzoic acid was obtained from Merck Chemical Co. (Germany). Tumor cells were obtained from Korean Cell Line Bank at Seoul National University. CBD was kindly provided by Prof. R. Mechoulam in the Department of Natural Products at School of Pharmacy, Hebrew University, Israel. All other chemicals were of reagent grade.

Cell culture

KB cells and NIH 3T3 cells were grown at 37°C in RPMI medium supplemented with 10% FBS penicillin (100units/ml) and streptomycin (100 μg/ml). The cells were grown in a humidified atmosphere of 95% air/5% CO_2. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytomer just before transferring them for the experiment.

Preparation of cannabigerol(1, CBG)

CBG (**1**) was identified by comparing its spectral data (TLC, MS, NMR and IR) with those published or by directly comparing it with an authentic sample (Baek *et al.*, 1996; Baek *et al.*, 1995).

Preparation of methyl 3-methoxy-4-hydroxybenzoate

3-Methoxy-4-hydroxybenzoic acid (10,610 g, 63 mmol) and sodium bicarbonate (8,100 g, 96 mmol) in acetone (50 ml) were heated under reflux with dimethyl sulphate (8.731 g, 69 mmol) for 48 h. After the reaction, the solvent was removed by TLC in vacuo to make a brown oil. Ice cold water (50 ml) was added and vigorously stirred for two hours to make a pale solid (9.7 g, 85%) which was collected by filtration. Found; R_f 0.75 (50% ether/hexane); mp 60-64°C. methyl 3-methoxy-4-hydroxybenzoate obtained was identified by comparing its spectral data (TLC, MS, IR, ^{1}H-NMR and ^{13}C-NMR) with those published or by directly comparing it with an authentic sample (Rama Roa *et al.*, 1981; May, 1995).

Preparation of methyl 4-[(2*E*)-3,7-dimethyl-2,6-octadienyl)oxy]-3-methoxybenzoate (Perry *et al.*, 1996)

DTM **2**, (380 mg, 60%) : yellow crystals; TLC R_f 0.31 (grey with vanillin/H_2SO_4) : mp 39-40 : calculated for $C_{19}H_{26}O_4$, C 71.70%, H 8.18%, found C 71.79%, H 8.31%. Methyl 4-[{(2*E*)-3,7-dimethyl-2,6-octadienyl}oxyl-3-methoxybenzoate obtained was identified by comparison of its spectral data (TLC, MS, IR, ^{1}H-NMR and ^{13}C-NMR) with those published or by direct comparison with an authentic sample(Baek *et al.*, 1998).

Evaluation of antitumor activity

The antitumor activities of CBG **1**, DTM **2**, 5-FU **3** and CBD **4** were determined by the modification of the literature methods (Mosmann, 1983; Carmichael *et al.*, 1987; Skehan *et al.*, 1987). All experimental data were expressed as the mean S.D. of triplicate experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT) assay

The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product, which can be measured spectrophotometrically (Mosmann, 1983; Carmichael *et al.*, 1987) KB cell lines were cultured in RPMI-1640 medium (Gibco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells (5×10^4) were cultured for 48 h at 37°C in a humidified 5% CO_2 incubator in the presence or absence of CBG **1**, DTM **2**, 5-FU **3** and CBD **4**.

Sulforhodamine B protein (SRB) assay

The SRB assay was performed essentially according to the method of Skehan *et al* (1990). The methods of plating and incubation of cells were identical to those of the MTT assay.

Evaluation of toxicity: Cytotoxicity assay

In order to determine the cytotoxicity mediated by CBG **1**, DTM **2**, 5-FU **3** and CBD **4**, the colorimetric assay was used. These compounds were serially diluted in EMEM (Eagle's minimum essential medium) with 10% FBS and mixed with equal volume of NIH 3T3 fibroblast (5×10^4 cells/ml). After one hour, fresh culture medium was supplied to a total volume of 1~100 μM. On the third day of incubation at 37°C an incubator MTT terazolium dye (5 mg/ml; 20 μl/well; Polyscience, Inc. Warrigton, PA) was added to the cells. After 3 h, the absorbance was measured at 540 nm using ELISA reader. All experimental data were expressed as the mean ± S.D. of triplicate experiments. The 50% cytotoxic dose (CD_{50}) was calculated using the computer program.

Morphology

Changes in the morphology of KB cells cultured in a medium with CBG **1**, DTM **2**, 5-FU **3** and CBD **4** were documented by microphotography.

Statistical analysis

All values, expressed as the mean ± S.D., were statistically analyzed through analysis of Student's t-test. The P value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

Antiproliferative activity

Table I shows the potent antitumor activities of CBG **1**, DTM **2**, 5-FU **3** and CBD **4** against KB cells. In general, the antitumor activities of these compounds were in a dose-dependent, and the susceptibility of the cancer cell lines to DTM (**2**) was quite sensitive. The value of IC_{50} of CBG (**1**), DTM **2**, 5-FU **3** and CBD **4** showed that DTM **2** exerts the most potent antitumor activity. The values of MTT_{50} and SRB_{50} were determined at 29.81 μM and 48.61 μM, respectively.

Table I. The antitumor activities of CBG, DTM, 5-FU, and CBD on KB cell lines. Comparison of IC_{50} for CBG, DTM, 5-FU and CBD by SRB assay and MTT assay

Compounds[a]	IC_{50} (μM)[b]	
	MTT assay	SRB assay
CBG	45.55	69.40
DTM	5.55	41.42
CBD	19.22	41.42
5-FU	44.36	45.20

a) Each compound was examined in four concentrations in triplicate experiments.
b) IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

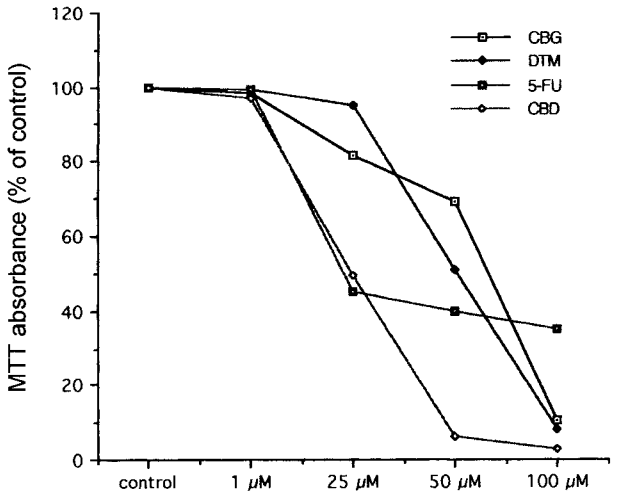


Fig. 1. *In vitro* cytotoxicities of CBG **1**, DTM **2**, 5-FU **3** and CBD **4** by MTT assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells). The colorimetric assay was performed as described in the materials and methods section. Data are mean values of results obtained from three sets of experiments.

Cytotoxicity

A colorimetric assay was used to detect the *in vitro* cytotoxicity mediated by DTM **2**. As shown in Fig. 1, DTM **2**-mediated cytotoxicity rapidly increased in the MTT assay when its concentration was increased from 25 μM to 100 μM. However, that of DTM **2** did not change in MTT assay when its concentration was raised from 1 μM to 25 μM (Fig. 5). As shown in Fig. 2, DTM (**2**)-mediated cytotoxicity rapidly increased in the MTT assay when its concentration was increased from 50 μM to 100 μM. However, that of DTM **2**, did not change in SRB assay when its concentration was increased from 1 μM to 50 μM (Fig. 2). The values of MTT_{50} and SRB_{50} were determined at 56.92 μM and 102.70 μM, respectively (Table II).

Synthesis of cannabigerol

CBG **1** is reported as a synthetic or a natural product. CBG 1 was synthesized by coupling olivetol to geraniol, which were prepared by literature methods (Baek *et al.*, 1996; Baek *et al.*, 1995; Baek *et al.*, 1985). The structure of CBG **1** was confirmed from spectral data.

In the previous study (Baek *et al.*, 1995; Baek *et al.*, 1985), we reported simple and convenient method for

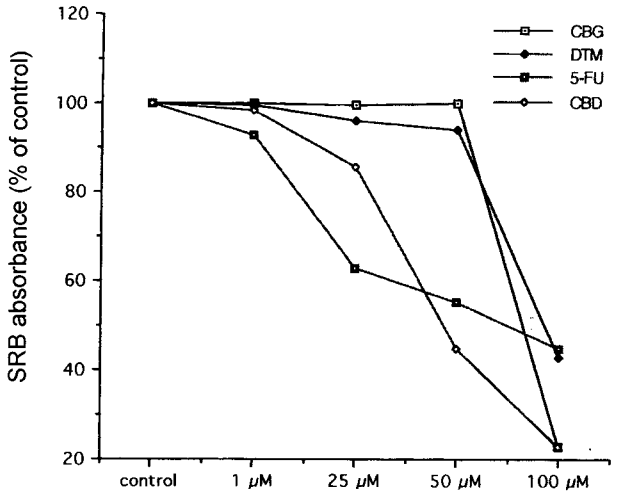


Fig. 2. *In vitro* cytotoxicities of CBG **1**, DTM **2**, 5-FU **3**, and CBD **4** by SRB assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells). The colorimetric assay was performed as described in the materials and methods section. Data are mean values of results obtained from three sets of experiments.

Table II. The cytotoxic effects of CBG, DTM, 5-FU and CBD on KB cell lines.Comparison of CD_{50} for CBG, DTM, 5-FU and CBD on NIH 3T3 fibroblasts by SRB assay and MTT assay

Compounds[a]	CD_{50} (μM)[b]	
	MTT assay	SRB assay
CBG	60.46	82.98
DTM	56.92	102.70
CBD	36.27	60.25
5-FU	41.27	75.90

a) Each compound was examined in four concentrations in triplicate experiments.
b) IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

creating the intermolecular Friedel-Crafts alkylation reaction in the synthesis of cannabigerol type cannabinoids from geraniol and olivetol in the presence of boron trifluoride-diethyl ether and silica in methylene chloride at room temperature for 2 days. The synthetic routes available were not practical for they lead to average yields of CBG. CBG **1** (C-geranyl compound) was prepared by using the standard synthetic route.

CBG **1**, a nonpsychoactive constituent, was identified by comparing its spectral data (TLC, MS, NMR, and IR) with those published or by directly comparing (Baek *et al.*, 1995; Baek *et al.*, 1985). When evaluated for its antitumor efficacy against melanoma cells of a mouse's skin, it showed significant activity with IC_{50}=31.30 μM/ (Kang *et al.*, 1985). Geraniol exhibited limited significant

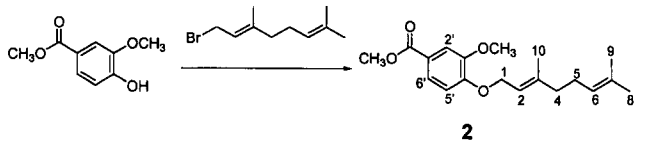
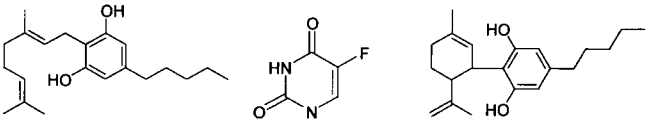


Fig. 3. Preparation of DTM 2



1 **3** **4**

Fig. 4. The structures of CBG(1), 5-FU (3) and CBD (4)

antitumor activity (Baek *et al.*, 1996, 1998; Baik *et al.*, 1988; Jung *et al.*, 1995). CBG **1** showed a low IC_{50} value of 31.30 μM.

Synthesis of methyl 3-methoxy-4-hydroxybenzoate

May (May, 1995) reported that methyl 3-methoxy-4-hydroxybenzoate (methyl vanillate) was shown to have mild cyto-toxicity but no antiviral or antitumor activity when tested for biological activity (Baek *et al.*, 1998).

Methyl vanillate is reported as a synthetic or a natural product. The synthesis of methyl vanillate resulted from coupling the 3-methyl-4-hydroxybenzoic acid to dimethylsulphate, which was prepared by literature methods (Asakawa, 1995). The structure of methyl 3- methoxy-4-hydroxybenzoate was confirmed from spectral data.

Synthesis of methyl 4-[(2*E*)-3,7-dimethyl-2,6-octadienyl)oxy]-3-methoxybenzoate

DTM **2** is reported as a natural product. The synthesis of deoxy-tomentellin **2** resulted from coupling geranyl bromide to methyl vanillate, which were prepared by literature methods (Baek *et al.*, 1998). The coupling of the isoprenyl unit to methyl vanillate was developed in the course of this project and gave DTM **2** in good yields (60%). We synthesized **2** (deoxy-tomentellin) by using an efficient and attractive method for synthesizing methyl 4-isoprenoxy-3-methoxybenzoate developed on a small scale (Fig. 3).

DTM **2** (*O*-geranyl compound) was prepared by using May's method (May,1995). DTM **2** (deoxy-tomentellin), the constituent of liverwort *Trichocolea tomentella*, was identified by comparison of its spectral data with those published or by direct comparison (Perry *et al.*, 1996; Baek *et al.*, 1998).

Perry *et al.* (1996) reported more isoprenyl benzoates from Japanese and European collections of *Trichocolea tomentella*, including DTM **2** (Asakawa *et al.*, 1981). Some of these compounds were also found in a

collection of *Trichocolea pluma* Mont. from Malaysia. 3,4-Dimethoxybenzoates were proposed as significant chemical markers of the *Trichocoleaceae* (Asakawa *et al.*, 1991). The ^{1}H NMR spectrum and IR spectrum were similar to those reported for DTM **2**. The ^{13}C NMR spectrum of our compound showed signals indicative of a trisubstituted aromatic ring, a geranyl group, an ester carbonyl, and two methoxyl groups, as expected for DTM **2**. We rigorously assigned the ^{1}H and ^{13}C NMR spectra with the aid of HMQC, HMBC, and NOE difference experiments. The NOE difference experiments produced a suprising result. Irradiation of the two methoxyl signals, which were barely resolved, only enhanced the H-2 aromatic proton signal. Structure **2** is expected to enhance both the H-2' and H-5' signals. An NOE interaction between H-5' and H-1 protons of the geranyl group was also consistent with structure **2**. DTM **2** was unequivocally verified from the HMBC experiment, which showed the relationships between the ester carbonyl (δ 167.0) and one methoxyl proton signal (δ 3.90, 3H) and between a quaternary oxygenated aromatic signal (δ 152.3) and the $H_{2\text{-}1}$ protons (δ4.68, d, J=7 Hz) of the geranyl group. A further NOE interaction between the geranyl H-10 and H-1 signals showed the *E* sterochemistry of the 2,3-double bond.

The present study also compares the *in vitro* growth inhibitory activities of DTM **2**, and 5-FU **3** and CBD **4** against KB cell lines. These compounds (**1, 2, 3** and **4**) were evaluated for antitumor efficacy against KB cells. The antitumor activities of these compounds (**1, 2, 3** and **4**) against KB cells are provided in Table I (Moon *et al.*, 1991; Woo *et al.*, 1996). DTM (*O*-geranyl compound) showed the most antitumor activity against KB cells (Table I).

Biological activity

The present study shows the *in vitro* growth inhibitory activities of cannabigerol **1**, DTM **2**, 5-FU **3** as a reference, and CBD **4** against KB cell lines. 5-FU is commonly used as a therapeutic agent to treat cancers of the large bowel. However, therapy with 5-FU **3** as a single agent has shown only limited success. Therefore, additive agents or modulators of the 5-FU are needed for a more effective treatment of these highly resistant malignancies (Boersma *et al.*, 1993).

The antitumor activities of these compounds **1, 2, 3** and **4** against KB cells are provided in Table (Moon *et al.*, 1991; Woo *et al.*, 1996). Table shows the potent antitumor activities of, CBG **1**, DTM **2**, 5-FU **3** as a reference, and CBD **4** against KB cells. In general, the antitumor activities of these compounds **1, 2, 3** and **4** were dose-dependent over the micromolar concentration range 1 to 100 μM, and the susceptibility of KB cells to these compounds varied sharply (Table I). A comparison of IC_{50} values of these compounds in tumor cell lines showed that their susceptibility to these compounds decreased in the following order: DTM > CBG > 5-FU > CBD by the MTT assay and DTM > CBG > CBD > 5-FU by the SRB assay (Table I). DTM **2** was the most effective growth inhibitor of KB cell lines, producing approximately 30 μM of IC_{50} in the MTT assay and 49 μM in the SRB assay. To test the KB cells for *in vitro* chemosensitivity the SRB assay was compared with the MTT assay. Having better linearity with the cell number and higher reproducibility, the MTT assay appeared to be more sensitive than the SRB assay.

Table II shows the cytotoxic activities of CBG **1**, DTM **2**, 5-FU **3** and CBD **4** against NIH 3T3 fibroblasts. In general, the cytotoxic activities of these compounds **1, 2, 3** and **4** were in a dose-dependent manner over the concentration range 1 μM to 100 μM, and the susceptibility of NIH 3T3 fibroblasts to these compounds varied. The comparison of CD_{50} values of these compounds in NIH 3T3 fibroblasts showed that their susceptibility to these compounds decreased in the following order ; CBD

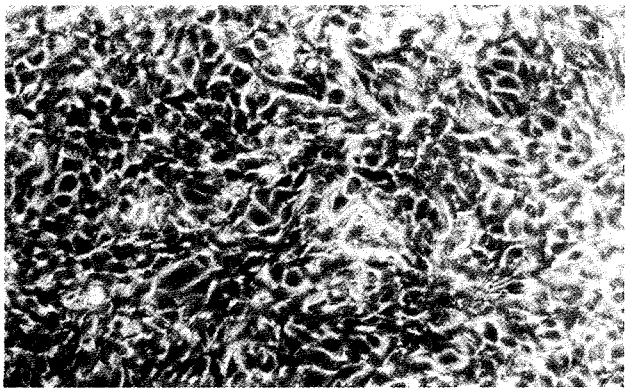


Fig. 5. Inverted photomicrograph of KB cells treated with MTT for an additional 3 h after incubation in unmodified medium (control) for 2 days × 400. Most cells had abundant cyptoplasm and cytoplasmic processes.

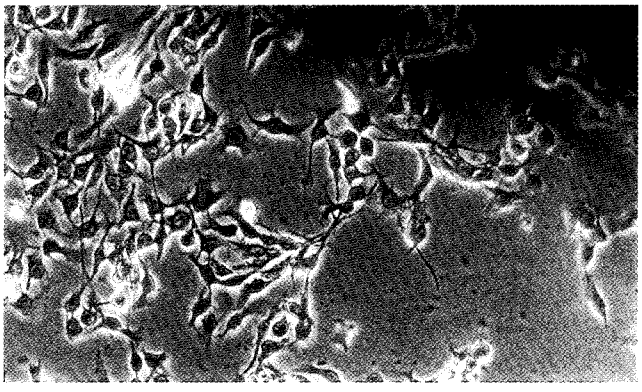


Fig. 6. Inverted photomicrograph of KB cells treated with MTT for an additional 3 h after incubation in 100 μM DTM 2 containing medium for 2 days × 200. Most cells formed cell clusters and number of cells was decreased.

> 5-FU > DTM > CBG by the MTT assay, CBD > 5-FU > CBG > DTM by the SRB assay (Table II). CBD **4** was structurally related to olivetol, which is known to have an inhibitory effect (Kim *et al.*, 1998), and exhibited the least effective growth-inhibitory activity against the tested cancer cell lines. As shown in Fig. 5 and 6, Most KB cells had abundant cyptoplasm and cytoplasmic process (Fig. 5). However, most cells were formed cell cluster and number of cells was decreased when CBD was raised to 100 μM (Fig. 6).

The compounds used are known to inhibit the activity of several enzymes of the arachidonate cascade including cyclo-oxygenase and lipoxygenase (Evans *et al.*, 1987) and to both stimulate and inhibit phospholipase A_2 activity (Evans *et al.*, 1987). The actions of the cannabinoids on membrane-associated enzymes are complex and dose-dependent. Although these actions may be related the anti-cancer ability of the cannabinoids (Plasse *et al.*, 1991), specific structural alterations may be critical in determining the enzyme targets. However, the antitumor activity of DTM **2** is more active than that of 5-FU **3** on KB cells. This compound **2** has been selected the primary compound for further examinations.

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