A New Clerodane Diterpenoid Isolated from Propolis

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A physiologically active substance has been isolated from Brazilian propolis and characterized as a new clerodane diterpenoid, as indicated by human hepatocellular carcinoma HuH 13 cell cytotoxicity assays. This compound inhibited growth of the hepatoma cells at a concentration around 10 μg/ml and arrested the tumor cells at S phase as revealed by flow cytometry. At higher concentrations it exerted lethal damage. The compound showed cytotoxicity on human lung carcinoma HLC-2, HeLa, KB and rat W3Y cells, whereas human diploid foreskin and primary rabbit kidney cells were less affected.

Introduction

Propolis is a folk medicine employed for treating various ailments. It is alleged to exhibit a broad spectrum of activities including antibiotic (Metzner *et al.,* 1979; Grange and Davey, 1990), antiinflammation (Wang *et al.,* 1993), antioxidant (Scheller *et al.,* 1990) and tumor cell arrest (Scheller *et al.,* 1989).

Ethyl ether extracts of propolis had previously been demonstrated to be cytotoxic to KB and HeLa cell lines (Hladoń *et al.,* 1980).

As assayed by the Ltk$^-$ cell growth inhibition test, Grunberger *et al.* (1988) isolated and characterized one such biologically active component as caffeic acid phenethyl ester.

Differential cytotoxicity has been reported in tumor and virally transformed cells *versus* normal ones in the presence of this substance (Su *et al.,* 1991; Guarini *et al.,* 1992; Frenkel *et al.,* 1993). Caffeic acid phenethyl ester was reported to be a lipoxygenase inhibitor with antioxidant properties (Sud'ina *et al.,* 1993). Inhibition of the tumor promoter-mediated oxidative processes by the compound has also been reported in HeLa cells (Bhimani *et al.,* 1993).

Rao *et al.* (1993) published that azoxymethane-induced colonic aberrant crypt foci formation, activities of ornithine decarboxylase, tyrosine protein kinase and lipoxygenase were significantly inhibited in rats fed caffeic acid phenethyl ester, indicating that the compound inhibited biochemical changes relevant to colon carcinogenesis.

We investigated chemical constituents of Brazilian propolis which showed tumoricidal activities. In the present paper we report the isolation and characterization of a new clerodane-type diterpenoid.

Materials and Methods

Extraction of propolis

Propolis, a gift of Nihon Propolis Co., Ltd., was collected from hives located in various districts of Brazil including São Paulo, Paraná and Santa Catarina *etc.* It was received in the form of dried powder which was homogenized for a few minutes with Polytron (Kinematica, Luzern, Switzerland) with ethyl acetate (5 v/w). The homogenate was suction-filtered and evaporated *in vacuo* to yield a golden brown solid. The yield was approximately 23% (w/w). The extract was dissolved in methanol and white precipitate formed was removed by centrifugation.

Cell cultures

Human hepatocellular carcinoma HuH 13 and primary rabbit kidney cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum and 10% tryptose phosphate broth at 37 °C under 95% air–5% CO_2. HeLa, KB, rat W3Y (fibroblast transformed by SV_{40} virus) and human diploid foreskin cells were cul-

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tured in Eagle's MEM containing 5% fetal calf serum. Human lung carcinoma HLC-2 cells (obtained from Tumour Laboratory, Kokubunji, Tokyo) were maintained in RPMI-1640 with 5% fetal calf serum.

Cytotoxicity assay

The methanol extract was fractionated by HPLC as described below and eluates were evaporated *in vacuo* and dissolved in dimethyl sulfoxide (DMSO). Human hepatoma HuH 13 cells were plated in 96 microwell plates (Falcon) with serially diluted test samples and cultured in Eagle's MEM with 10% calf serum. Cell growth inhibition or damaging activities of the materials were routinely determined by visual inspection under microscope after 3-days incubation period. The final concentrations of the solvent were less than 2% to avoid the cell damage.

Cytotoxic effects of the clerodane diterpenoid on various cells were determined by the tetrazolium salt MTT assay according to the method of Mosmann (1983). In brief, cells were plated in the microplates and cultured in the media with the serially diluted compound dissolved in DMSO and incubated for 3 days. After removal of the culture media and dead cells, the residual cells were incubated with fresh media containing MTT and blue formazan produced was measured at 570 nm on an ELISA reader.

Purification of a cytotoxic substance

The methanol extract of propolis was applied to an ODS 80 T_M column (2.5×34 cm, Toso Co., Ltd.) and eluted with a linear gradient of 50–90% (v/v) methanol. Fractionation of the extract by the reverse phase HPLC yielded many active fractions lethal to the hepatoma cells. Fractions with 82–88% (v/v) methanol were collected. Caffeic acid phenethyl ester was eluted from the column with lower concentrations of methanol (around 64% (v/v)). After concentration *in vacuo,* the eluate dissolved in 90% acetonitrile (v/v) were adsorbed on the column and desorbed with a linear gradient of 70–100% (v/v) acetonitrile at the concentrations about 73–76% (v/v). The substances with slightly different resins which had no tumoricidal activities were removed by a final passage through the column with a linear gradient of 75–90% (v/v) ethanol. As shown in Fig. 1 two peaks were detected absorbing ultraviolet light (254 nm). A tumoricidal substance was obtained by collecting the posterior peak fractions. Final yield (20 mg) was about 0.03% with respect to the ethyl acetate extract. There was no evidence that another lethal constituent had been separated by the purification steps after the chromatography with methanol as eluant.

Retention time of the purified material on a reverse phase silica gel column (6.0×150 mm, AM-312, YMO Co., Ltd.) was 16.7 min (eluant: 0.02 M acetic acid–ammonium acetate (pH 5.0)–methanol [25:75 (v/v)]; flow rate: 1 ml/min; detected by means of 240 nm ultraviolet absorption.

Flow cytometry

HuH 13 cells (approximately 10^6) were trypsinized, washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and fixed in 70% ethanol, then the cells were incubated at room temperature for 30 min in a porpidium iodide-staining solution (50 μg/ml in Ca^{2+}/Mg^{2+}-free PBS, pH 7.4). DNA analysis was performed with a FACScan flow cytometer (Becton Dickinson,

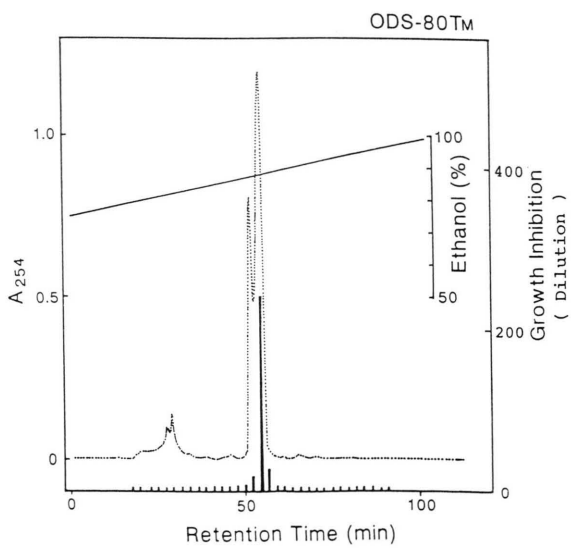

Fig. 1. Chromatographic pattern of the eluate obtained by fractionation with acetonitrile. 1 ml aliquots of the eluate in the fraction tubes were dried *in vacuo* and dissolved in 50 μl of DMSO. The cell growth inhibition and cell damaging activities were determined as described in Materials and Methods.

San Jose, CA) coupled with a Hewlett-Packard computer.

Physicochemical analyses

NMR spectrum was measured with Bruker AM 500 nuclear magnetic resonance apparatus and mass spectrum with Hitachi M-BOB and JEOL SX-102 spectrometer by Dr. M. Yoshida and Dr. Y. Saito (Tokyo Institute, Kyowa Hakko Co., Ltd.), respectively.

Results and Discussion

Molecular structure of the compound

The structure of this colorless component was determined as being a new clerodane diterpenoid (named PMS-1) as shown in Fig. 2 with a molecular formula $C_{20}H_{33}O_3$ (MW: 320) deduced from the following data.

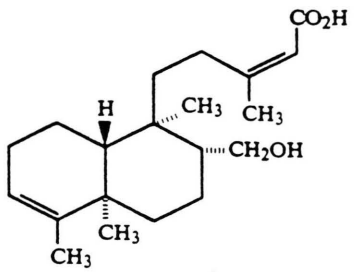

Fig. 2. Chemical structure of a new clerodane diterpenoid (PMS-1) isolated from Brazilian propolis.

1. Mass spectrum *m/z:* 321 $(M+H)^+$ (by SIMS method using glycerol as a matrix). High resolution SIMS spectrum: 321.2403 (calculated as $C_{20}H_{33}O_3$: 321.2430).

2. Infra-red absorption spectrum (KBr tablet method): ν (cm^{-1}): 3600–2400, 3450, 2941, 1695, 1641, 1439, 1385, 1259.

3. ^1H NMR (500 MHz, CD_3OD): internal standard TMS, δ (ppm): 5.63 (1H, br, s), 5.18 (1H, m), 3.84 (1H, dd, J = 10.6, 3.1 Hz), 3.21 (1H, br, t), 1.89 (3H, d, J = 1.3 Hz), 1.58 (3H, d, J = 1.3 Hz), 1.03 (3H, s), 0.75 (3H, s).

4. ^{13}C NMR (125 MHz, CD_3OD): internal standard TMS, δ (ppm): 170.4, 161.7, 145.1, 121.8, 117.7, 64.4, 47.9, 45.5, 39.4, 39.2, 37.7, 37.6, 28.4, 27.7, 25.4, 23.2, 20.3, 19.5, 18.8. 18.2.

5. Ultraviolet absorption spectrum (in methanol): λ_{max}: 212 nm (ε = 4,600), 288 nm (ε = 2,600).

6. Specific rotary power: $[\alpha]_D^{25} = -83°$ (c = 0.44, methanol).

Effect of PMS-1 on the cell cycle

As shown in Fig. 3 initial experiments using flow cytometry to examine DNA content in non-synchronized hepatoma HuH 13 cells revealed that fractions in G_1/G_0 and G_2/M phases decreased with concomitant increase in S phase fractions

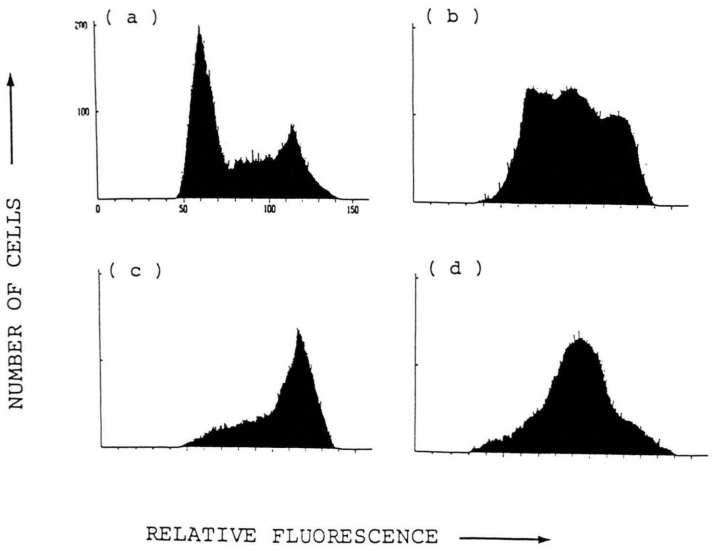

Fig. 3. Flow-cytometric analysis of cellular DNA content in HuH 13 cells. (a) Control; (b) with clerodane diterpenoid (10 μg/ml); (c) with colcemid (2 μg/ml); (d) with colcemid and clerodane diterpenoid.

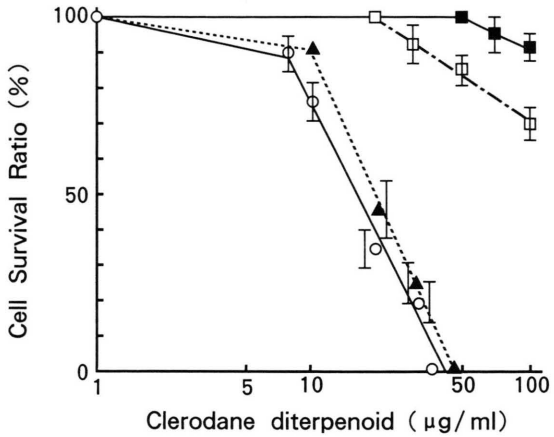

Fig. 4. Cytotoxic effect of a clerodane diterpenoid on various cultured cells *in vitro*. ○, Human hepatoma HuH 13; ▲, human lung carcinoma HLC-2; □, human diploid foreskin; ■, primary rabbit kidney cells. Each value indicates the mean (± S.D.) of triplicate experiments.

when the cells were exposed to PMS-1 (10 μg/ml) for 20 h.

For a more precise examination of where in the cell cycle the substance may be acting to inhibit cell cycle progression, the cells were incubated with PMS-1 in the presence of colcemid (2 μg/ml), an inhibitor of cell division. Cell population treated with PMS-1 largely had rather broad spectrum of DNA content characteristic of S phase.

These results indicate that the compound inhibits progression of the cells through S phase but not completion of M phase, suggesting that it suppresses the cellular DNA synthesis.

Cytotoxicity of PMS-1

When HuH 13 cells were exposed to the compound in the concentrations above 20 μg/ml the cells were damaged. The cells swelled and appeared more granulated with a rougher surface.

Time to cell death by exposure of the cells to the compound was inversely related to dose.

Cytotoxic effect of PMS-1 on human hepatoma HuH 13, human lung carcinoma HLC-2, human diploid foreskin and primary rabbit kidney cells cultured *in vitro* is presented in Fig. 4. Susceptibility of HeLa, KB and rat W3Y cells to the compound was similar to that of HuH 13 cells (data not shown). Less cytotoxic effects of the substance were observed on the confluent monolayers of non-transformed primary rabbit kidney cells and human diploid cells. We are now examining the cytotoxicity of the compound on various cultured cells.

Preliminary experiments showed that intracellular Ca^{2+} was elevated by exposure of HuH 13 cells to the compound. However, cell damage was also induced by the compound in the culture medium without Ca^{2+}.

The mechanism of cell death relating to the processes of necrosis and apoptosis has been reviewed (Fawthrop *et al.*, 1991). The first effect of intoxication in the mammalian cells may be due to perturbation of permeability for ions and small molecules through the cell membrane. The precise mechanism of the cytotoxicity observed and physiological activities of this new substance are under investigation in relation to the cell membrane structure and function.

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