

Hormetic and Anti-radiation Effects of Tropolone-related Compounds

KAORI KANTOH¹, MANAMI ONO¹, YUKO NAKAMURA², YUKIO NAKAMURA²,
KEN HASHIMOTO³, HIROSHI SAKAGAMI³ and HIDETSUGU WAKABAYASHI¹

¹Faculty of Science, Josai University, Sakado, Saitama, Japan;

²Division of Endodontics and ³Division of Pharmacology,
Meikai University School of Dentistry, Sakado, Saitama, Japan

Abstract. We have previously investigated a total of 173 azulene-, tropolone- and azulenequinone-related compounds for their tumor-specificity and anti-inflammatory activity. In this study, we selected six compounds that showed tumor-specific cytotoxicity (referred to as group I compounds) and five compounds that inhibited nitric oxide production by activated macrophages (referred to as group II compounds) to investigate their possible hormetic and anti-radiation effects. We have established three oral normal cell type, human gingival fibroblast HGF-1, pulp cell HPC-1 and periodontal ligament fibroblast HPLF-1, from extracted teeth and periodontal tissue. These normal cells expressed p53 protein, regardless of the growth stage (either at growing or near confluent phase), more than oral squamous cell carcinoma cell line (HSC-2). Group I compounds slightly stimulated the growth of HPL-1 cells only at restricted durations and concentrations, but did not affect that of HGF-1 and HPC-1 cells, suggesting the minor hormetic effects displayed by these compounds. We established a new evaluation system for UV-induced cellular damage using an intact HSC-2 cell system in which sodium ascorbate (vitamin C) and gallic acid, but not N-acetyl-L-cysteine nor catalase, exerted protective effects. Three group I compounds and two group II compounds significantly protected the cells from UV-induced injury, suggesting their possible anti-UV effect.

Heterocyclic compounds are known to display diverse biological activities (1). Hinokitiol and its related derivatives with a tropolone skeleton (2-4) have been reported to exhibit various biological activities such as antimicrobial (5),

Correspondence to: Dr. Hidetsugu Wakabayashi, Faculty of Science, Josai University, Sakado, Saitama 350-0295, Japan. Tel: +81 0492717959, Fax: +81 0492717985, e-mail: hwaka@josai.ac.jp / sakagami@dent.meikai.ac.jp

Key Words: Tropones, hormesis, UV protection.

antifungal (6) and phyto-growth-inhibitory activity (7, 8), cytotoxic effects on mammalian tumor cells (9, 10), and inhibitory effects on catechol-O-methyltransferase (11) and metalloproteases (5).

We have recently investigated a total of 173 azulene-, tropolone- and azulenequinone-related compounds for their tumor-specificity and anti-inflammatory activity. Among these compounds, 2,3-dimethyl-1-trichloroacetylazulene [**5b**], 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [**11b**] (12), 6,8,10-tribromobenzo[*b*]cyclohepta[*e*][1,4]oxazine [**9**], 6-bromo-2-chlorobenzo[*b*]cyclohepta[*e*][1,4]oxazine [**21**] (13), 7-bromo-2-(4-hydroxyanilino)tropone [**16**] and 2-(2-hydroxyanilino)-4-isopropyltropone [**20**] (14), tentatively classified as group I compounds (Figure 1), showed higher cytotoxicity against human oral squamous cell carcinoma (OSCC) cell lines (HSC-2, HSC-3, HSC-4) as compared with normal oral human cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)], yielding a tumor-specificity index (TS) of >35.6, >44.1, 12.5, 11.5, 4.0 and 4.4, respectively (Table I). On the other hand, benzo[*b*]cyclohepta[*e*][1,4]thiazine [**1**], 6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine [**2**] (15), benzo[*b*]cyclohepta[*e*][1,4]oxazin-6(11*H*)-one [**5**] (16), 3-methyl-1-trichloroacetylazulene [**2b**] and 3-ethyl-1-trichloroacetylazulene [**3b**] (17), tentatively classified as group II compounds (Figure 1), inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like RAW264.7 cells, with a selectivity index (SI) of >463.0, 18.4, 74.9, >37.7 and >36.1, respectively (Table I).

It has been reported that many toxic substances, environmental hormones, inorganic compounds, and even irradiation, modulate the growth of cultured cells in a biphasic fashion, stimulating or inhibiting growth at lower and higher concentrations, respectively. This growth-stimulating effect at lower concentrations is known as hormesis (18, 19). However, we recently found that three Chinese herbal extracts (from *Drynaria baronii*, *Angelica sinensis* and *Cornus officinalis* Sieb. et Zucc) failed to induce hormesis in human oral carcinoma cell lines (20). We also reported that

Table I. Tumor-specificity and anti-inflammatory activity of compounds used in this study.

	Tumor-specific cytotoxicity (TS)	Inhibition of LPS-stimulated NO production by macrophages (SI)	Ref.
Group I			
2,3-Dimethyl-1-trichloroacetylazulene [5b]	>35.6		12
1,3-Ditrichloroacetyl-4,6,8-trimethylazulene [11b]	>44.1		12
6,8,10-Tribromobenzo[b]cyclohepta[e][1,4]oxazine [9]	12.5		13
6-Bromo-2-chlorobenzo[b]cyclohepta[e][1,4]oxazine [21]	11.5		13
7-Bromo-2-(4-hydroxyanilino)troponone [16]	4.0		14
2-(2-Hydroxyanilino)-4-isopropyltroponone [20]	4.4		14
Group II			
Benzo[b]cyclohepta[e][1,4]thiazine [1]		>463.0	15
6,8-Dibromobenzo[b]cyclohepta[e][1,4]thiazine [2]		18.4	15
Benzo[b]cyclohepta[e][1,4]oxazin-6(11H)-one [5]		74.9	16
3-Methyl-1-trichloroacetylazulene [2b]		>37.7	17
3-Ethyl-1-trichloroacetylazulene [3b]		>36.1	17

TS values were determined by dividing the 50% cytotoxic concentration (CC₅₀) against normal cells by that against tumor cells. SI values were determined by dividing the CC₅₀ by EC₅₀ (concentration that reduced the LPS-induced NO production by 50%).

sodium fluoride failed to induce hormesis during the aging process of HGF, HPC and HPLF, suggesting the importance of duration of treatment in inducing hormesis (21). This was supported by our recent finding that [16] and [20] stimulated the growth of normal skin and lung human fibroblasts most prominently at 24 hours, but the stimulatory effects rapidly disappeared with prolonged incubation (48-96 hours), suggesting the occurrence of a hormetic response only at certain durations and concentrations (22). To confirm the generality of the occurrence of hormesis, we investigated whether six group I compounds, including [16] and [20], induce hormesis in newly established human normal oral cells (HGF-1, HPC-1 and HPLF-1).

We report here the establishment of a new evaluation system for UV-induced cellular damage using an intact cell system in which sodium ascorbate (vitamin C) and gallic acid, but not *N*-acetyl-L-cysteine (NAC) nor catalase, exerted protective effects. Using this newly established method, we also investigated here whether group I and II compounds protect cells from UV-induced cellular injury.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), α -MEM (GIBCO BRL, Gland Island, NY, USA); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), catalase, *N*-acetyl-L-cysteine (NAC) (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan); sodium ascorbate, gallic acid (Tokyo Chemical Industry Co., Ltd., Tokyo).

Synthesis of test compounds. Group I and II compounds were prepared as described previously (12-17).

Cell culture. HGF-1, HPC-1 and HPLF-1 cells were established from the first premolar extracted tooth in the lower jaw (because of dysfunctional position or orthodontic treatment), and periodontal tissues of a twelve-year-old girl, according to the guideline of Institutional Board of Meikai University Ethic Committee (No. A0808) after obtaining informed consent from the patients. These cells were cut into small pieces by surgical blade, and placed onto 80-mm plastic dish (Becton Dickinson Labware, NJ, USA). Cells were incubated with 2 ml of α -MEM supplemented with 20% heat-inactivated FBS for 2 weeks to allow the outgrowth of the cells. The addition of small amounts of culture medium to the cells was crucial to prevent their detachment from the dish by buoyancy. The outgrown cells were used as primary culture with population doubling level (PDL) zero. Cells were then harvested by treatment with 0.25% trypsin-0.025% EDTA-2Na in phosphate buffer without calcium and magnesium [PBS(-)]. The number of viable cells that excluded trypan blue was determined by hemocytometer under light microscopy and the total number of viable cells ($A_1 \times 10^4$ /dish) was calculated. One fourth of cells [$(1/4) \times A_1 \times 10^4$] were taken and supplemented with 3 times the amount of medium and cultured for one week with one medium change. When cells reached the second confluent phase (2 PDL), they were harvested, and one-fourth of them were similarly inoculated, and cultured with medium change in between. This procedure was repeated until cells stopped dividing. Given the total number of subcultures as *n*, the life-span of the cells is 2*n*; the cumulative cell population doubling number is then $A_1 \times 10^4 \times 2^n$.

OSCC cell lines (HSC-2, HSC-3, HSC-4) were cultured as described previously (12-14, 22).

Assay for cytotoxic activity. Cells were inoculated at 1×10^3 cells/0.1 ml in the inner 60 wells of a 96-microwell plate (Becton Dickinson Labware). The surrounding 36 exterior wells were filled with 0.1 ml

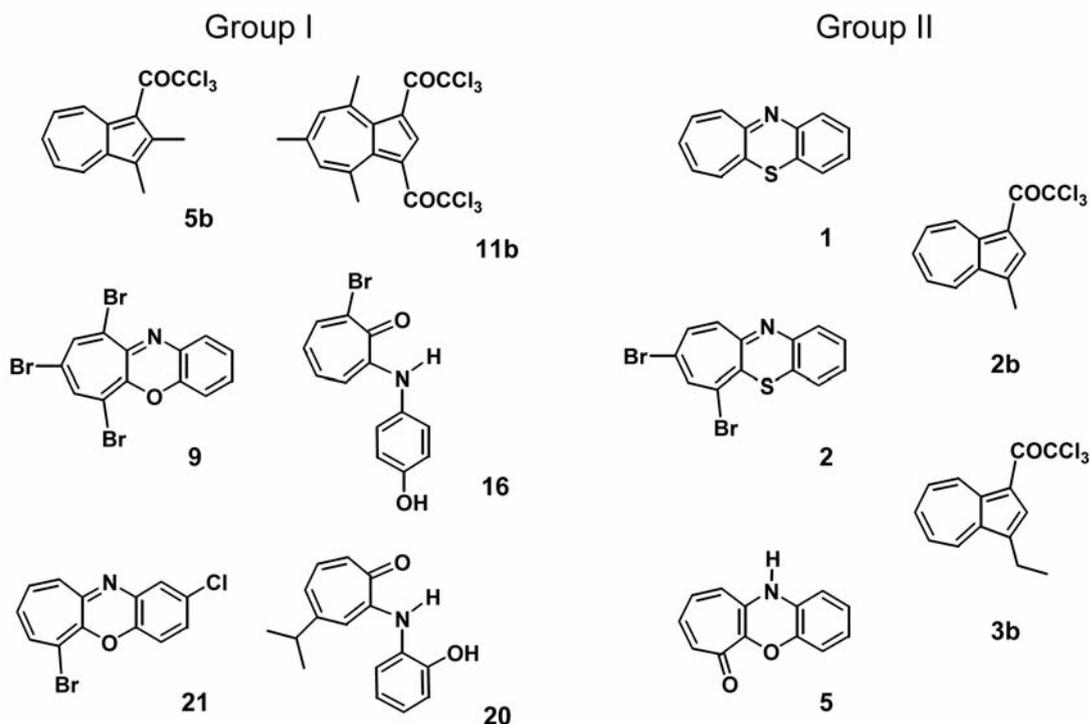


Figure 1. Chemical structure of group I compounds: 2,3-dimethyl-1-trichloroacetylazulene [5b], 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b], 6,8,10-tribromobenzo[b]cyclohepta[e][1,4]oxazine [9], 6-bromo-2-chlorobenzo[b]cyclohepta[e][1,4]oxazine [21], 7-bromo-2-(4-hydroxyanilino)tropolone [16], 2-(2-hydroxyanilino)-4-isopropyltropolone [20], and Group II compounds: benzo[b]cyclohepta[e][1,4]thiazine [1], 6,8-dibromobenzo[b]cyclohepta[e][1,4]thiazine [2], benzo[b]cyclohepta[e][1,4]oxazin-6(11H)-one [5], 3-methyl-1-trichloroacetylazulene [2b], 3-ethyl-1-trichloroacetylazulene [3b].

of PBS (-) to minimize the evaporation of water from the culture medium. After 48 hours, the medium was removed by suction with aspirator, and replaced with 0.1 ml of fresh medium containing different concentrations of single test compounds. The first well (500 μ M, sometimes 250 or 125 μ M) was diluted 2-fold sequentially, with triplicate wells for each concentration. Cells were incubated for 24, 48 or 72 hours, and the relative viable cell number was then determined by MTT method. In brief, the treated cells were incubated for another 4 hours in a fresh culture medium containing 0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of DMSO, and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labssystem, Helsinki, Finland).

Assay for hormesis. The hormetic response was evaluated by the maximum response in each dose-response curve, as described previously (18, 19, 22).

Assay for UV protection. The cells attached to 96-microwell plates were washed with PBS(-) and then replenished with DMEM+10%FBS or PBS(-). The cells were then placed at 21 cm distance from a UV lamp (wavelength: 253.7 nm) and exposed to UV irradiation (6 J/m²/min) for different durations (0-2 min) (Figure 2). The media were replaced with fresh DMEM+10%FBS and cells again cultured at 37°C in a CO₂ incubator until 24 hours after the start of irradiation.

Assay for BMI-1 and p53 protein expression. The cell pellets were lysed with 50 μ l of lysis buffer [10 mM Tris-HCl (pH 7.6), 1% Triton[®] X-100, 150 mM NaCl, 5 mM EDTA-2Na, 2 mM phenylmethylsulfonyl fluoride and 1 \times protease inhibitor cocktail] for 10 minutes on ice. The cell lysates were centrifuged at 16,000 \times g for 20 minutes at 4°C to remove the insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from cell lysates (30 μ g) was mixed with 2 \times sodium dodecylsulfate (SDS) sample buffer [0.1 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol], boiled for 5 minutes, and applied to SDS-10% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in PBS (-) plus 0.05% Tween 20 for 90 minutes and incubated for 60 minutes at room temperature with anti-BMI-1 (cone F6; Millipore Co., Billerica, MA, USA) (dilution: 1:2,000), or anti-p53 (DO-1: sc-126; Santa Cruz Biotechnology, Delaware, CA, USA) (dilution: 1:2,000), or anti-actin antibody (1:2,000, Sigma), and then incubated with horseradish peroxidase-conjugated anti-mouse IgG for 60 minutes at room temperature. Immunoblots were developed with Western Lighting[™] Chemiluminescence Reagent Plus (17).

Statistical treatment. The difference between two groups was evaluated by Student's *t*-test.

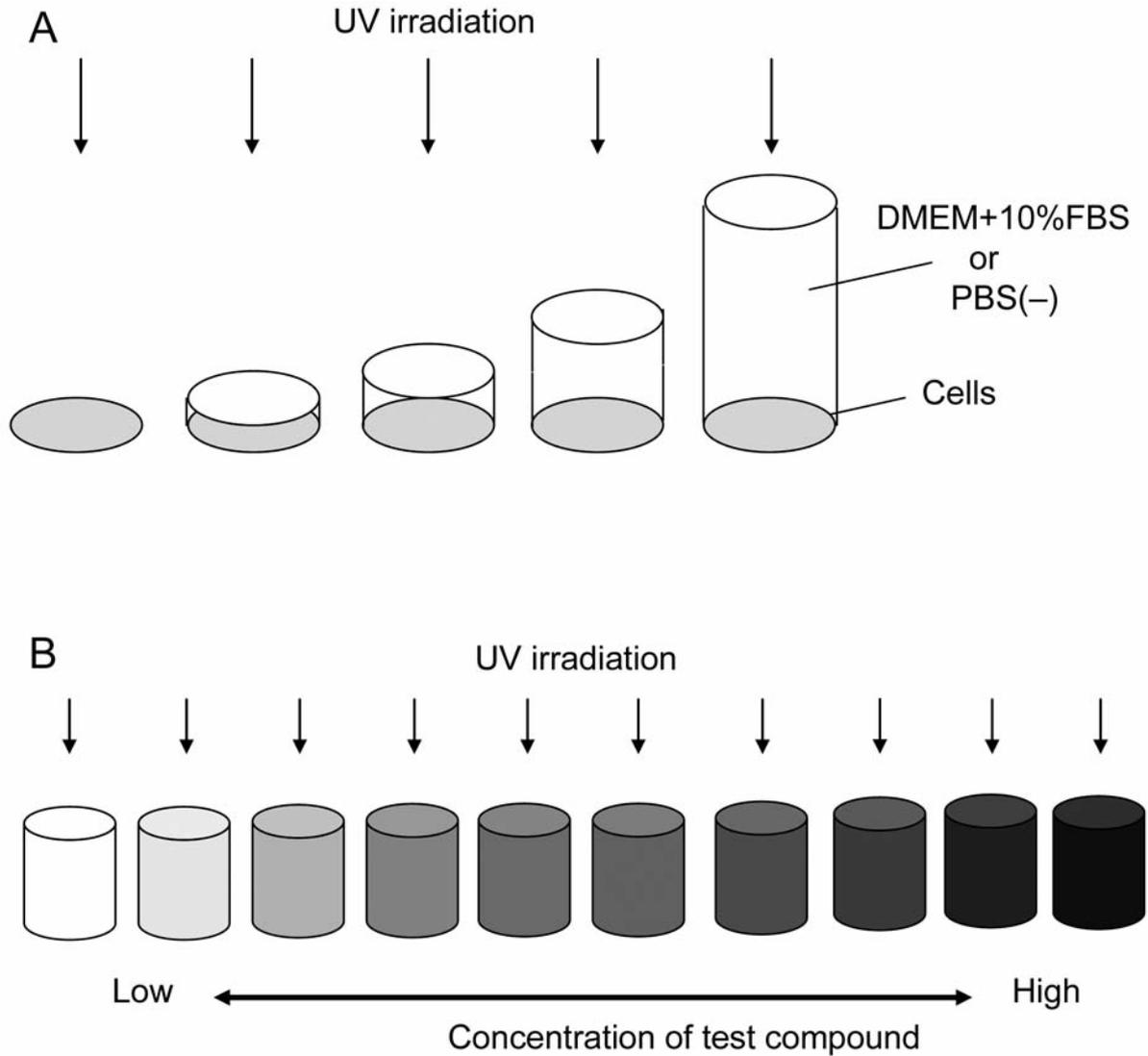


Figure 2. Experimental protocols for UV irradiation. Effect of the amount of medium (A) and the concentration of test compound (B).

Results

Culture conditions. We first determined which media, either DMEM or α -MEM, was most suitable for culture of human normal oral cells. HGF-1, HPC-1 and HPLF-1 had an *in vitro* life span (cumulative cell population doubling number) of 47, 43 and 43 PDL, respectively, regardless of the culture medium used (Figure 3A, B, C). The saturation density of the cells declined with *in vitro* senescence in both media, but was higher in DMEM/10%FBS than α -MEM/10% FBS (Figure 3D, E, F). During one weeks culture, cells in DMEM/10%FBS had a slightly, but not significantly (only two points for HPLF-1 (I) were $p < 0.05$) higher saturation

density than those in α -MEM/10%FBS (Figure 3G, H, I). Based on this finding, we used DMEM/10%FBS in the subsequent experiments.

All human oral cells (HGF-1, HPC-1, HPLF-1) expressed comparable amounts of p53 protein, regardless of the growth stage (either at growing or near confluent phase). On the other hand, the OSCC cell line (HSC-2) expressed negligible amounts of p53 protein at both phases (Figure 4). HSC-3 expressed p53 protein only at confluent phase, but not growth phase. HSC-4 cells do not express detectable level of p53 protein at both phases (data not shown). Our repeated experiments demonstrated that the expression of BMI-1 protein in these cells are below the detection level (data not shown).

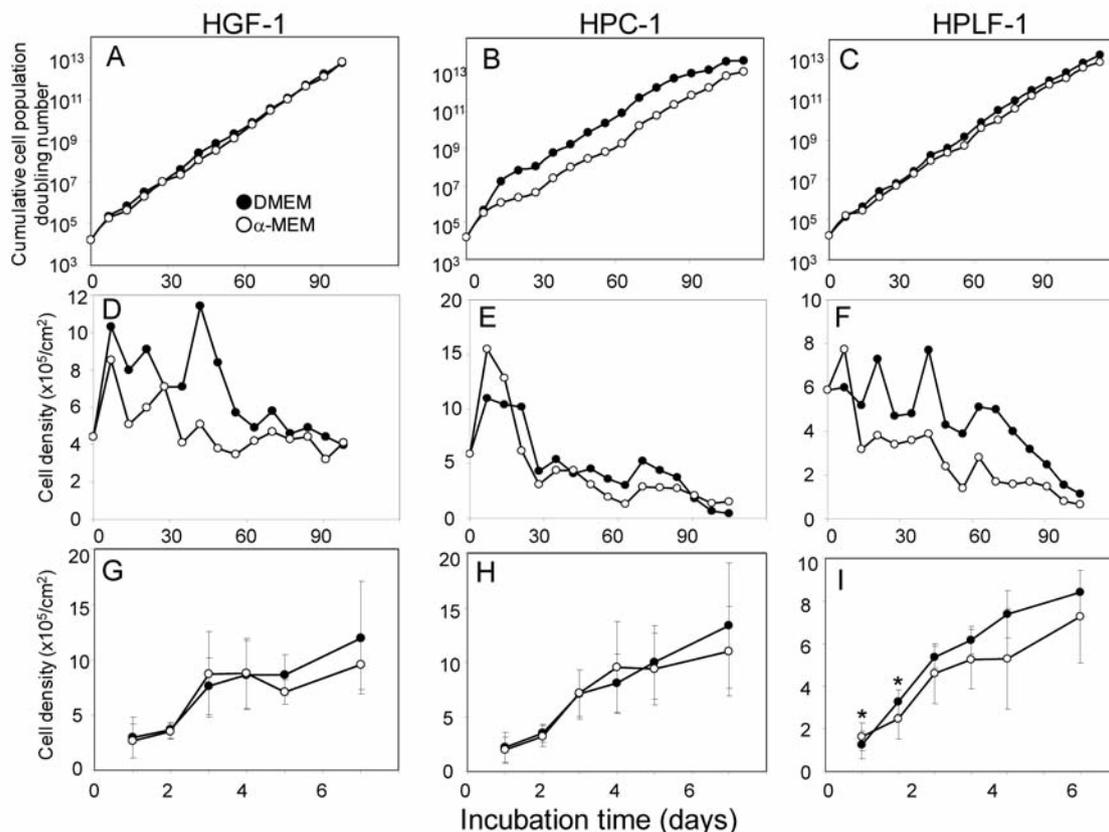


Figure 3. Effect of culturing in DMEM or α -MEM supplemented with 10%FBS on cumulative cell population doubling number (A-C), saturation cell density during the aging process (D-F) and growth curve (G-I) of HGF-1 (A, D, G), HPC-1 (B, E, H) and HPLF-1 (C, F, I) cells. Each value represents the mean of 6 determinations. Significant difference ($p < 0.05$) between DMEM and α -MEM is shown by asterisks in HPLF-1 (I).

Hormetic response. Since group I compounds, but not group II compounds, showed higher tumor-specificity, we investigated whether the hormetic response may be involved in the higher tumor specificity of group I compounds. When HPLF-1 cells were incubated for 24, 48 or 72 hours with group I compounds, slight growth stimulation was observed at 24 hours, but not at 48 or 72 hours. Such hormetic effects were not observed in HGF-1 nor HPC-1 cells, regardless of the incubation time (Figure 5).

Protective effects against UV irradiation. We first set up the experimental condition for UV irradiation (Figure 2). Human normal cells (HGF-1, HPC-1, HPLF-1) and human OSCC cell lines (HSC-2, HSC-3, HSC-4) were cultured without or with different volumes of culture medium (0-0.4 ml) and exposed to UV irradiation (Figure 6A). Cytotoxic effect of UV irradiation on OSCC cell lines was weakened by increasing amounts of culture medium, suggesting the presence of radical scavenger(s) or UV absorbing substance(s) such as phenol red and proteins in the culture

medium. On the other hand, normal cells were much more resistant to UV irradiation, as compared with OSCC cell lines, regardless of the amount of culture medium used (Figure 6A). When culture medium was replaced by PBS(-), cytotoxicity of UV irradiation against OSCC cells was enhanced (Figure 6B), possibly due to radical generation in PBS(-). Normal cells were again resistant to UV irradiation in PBS(-) (Figure 6B).

We next investigated the protective effects of increasing concentrations of group I and II compounds against UV-induced cellular damage (Figure 7). We used the HSC-2 cells for this experiment, since normal cells were much less sensitive to UV irradiation and HSC-2 cells were most sensitive among three OSCC cell lines (Figure 6). Among six group I compounds, [16] most potently reduced the cytotoxicity of UV irradiation, followed by [20] and then [11b]. [5b], [9] and [21] were relatively inactive (Figure 7A). Among five group II compounds, [5] was most protective against UV damage, followed by [2]. [1], [2b] and [3b] were inactive (Figure 7B). Among popular antioxidants, sodium

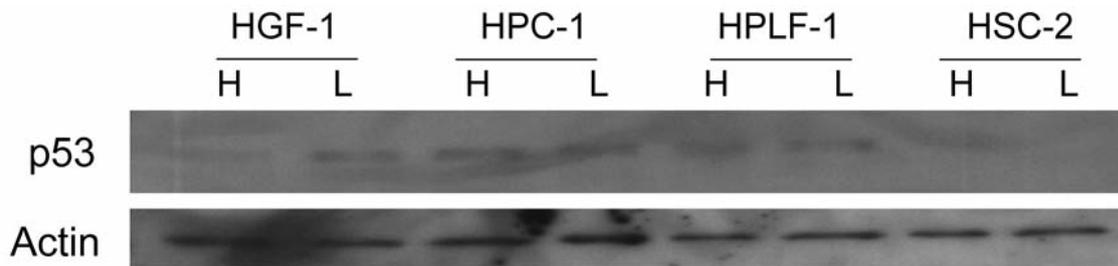


Figure 4. Expression of p53 proteins in human normal and OSCC cells. Cell lysates were prepared from human oral normal cells (HGF-1, HPC-1, HPLF-1) (20 PDL) or human OSCC cell lines (HSC-2) at low cell density (L) (growing phase) or high cell density (H) (near confluent phase), and then aliquots (equivalent to 30 μ g protein) were applied to Western blot analysis. The absence of p53 protein in HSC-2 cells was reproducible in another independent experiment.

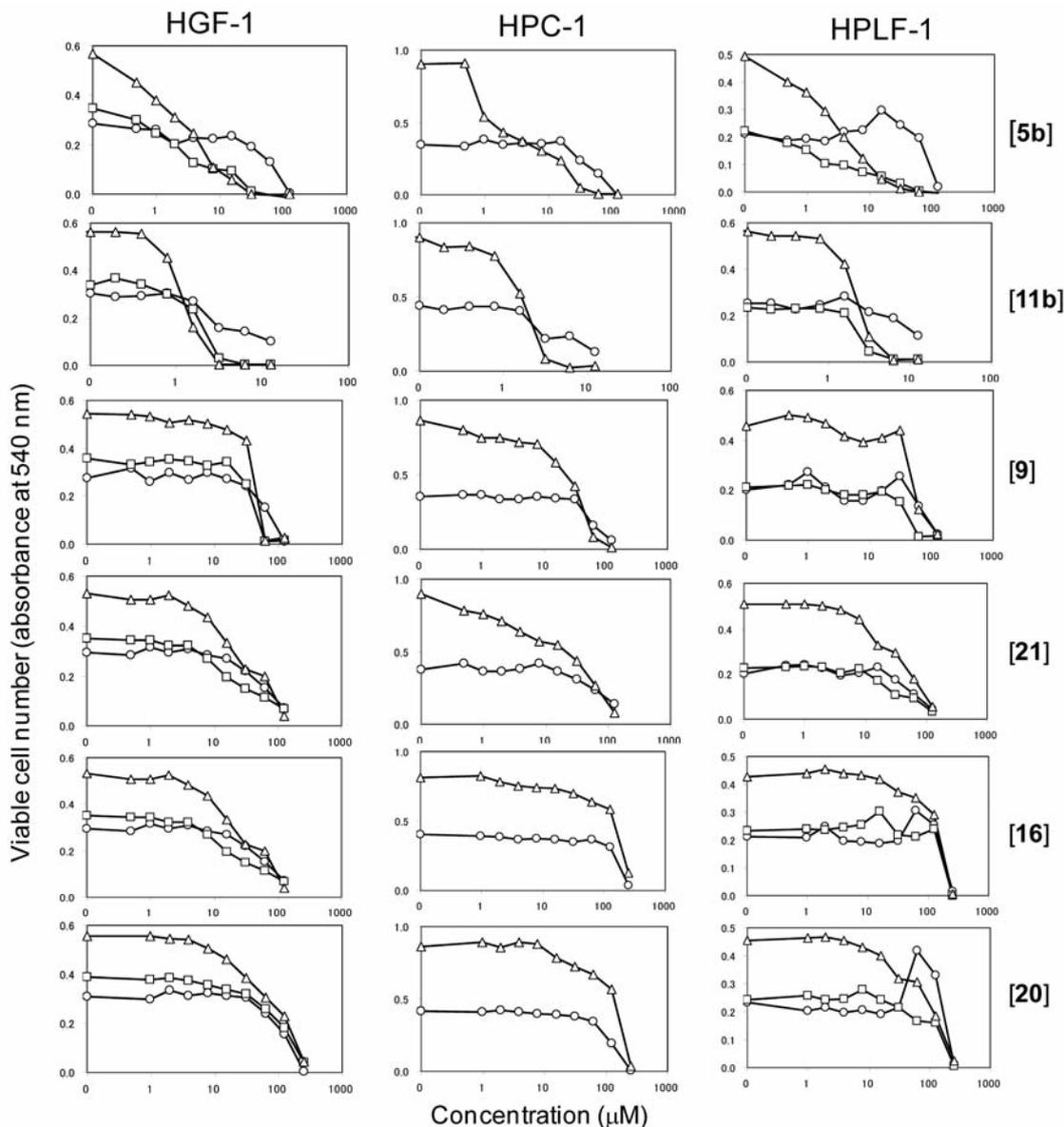


Figure 5. Hormetic effects of group I compounds. HGF-1 (23 PDL), HPC-1 (24 PDL) and HPLF-1 (24 PDL) cells were treated for 24 (\circ), 48 (\square) or 72 (\triangle) hours with [5b], [11b], [9], [21], [16] or [20], and the viable cell number was determined by MTT method. Each value represents the mean of three determinations.

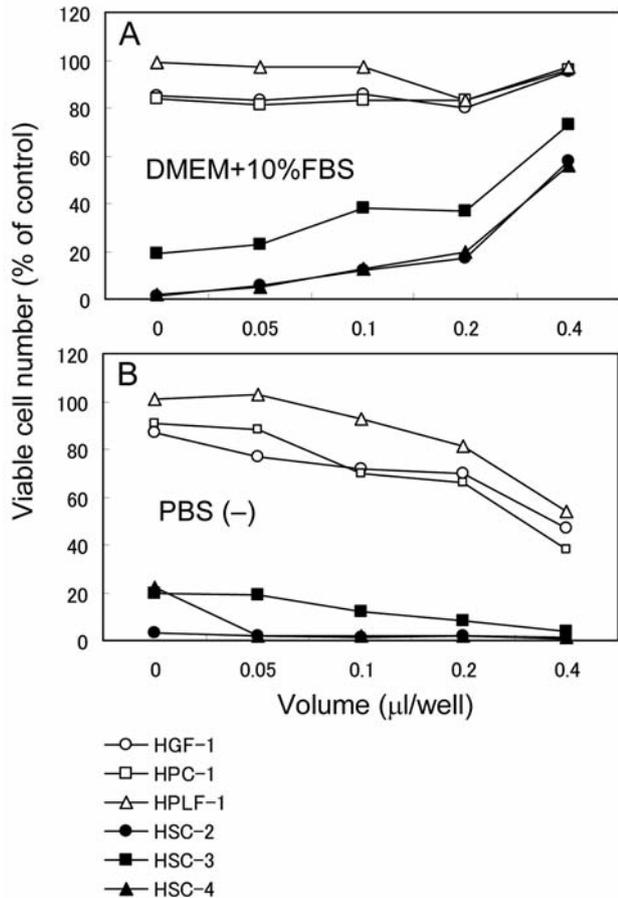


Figure 6. HGF-1 (15 PDL) (○), HPC-1 (16 PDL) (□), HPLF-1 (15 PDL) (△), HSC-2 (●), HSC-3 (■) or HSC-4 (▲) cells were exposed to UV irradiation (6 J/m²/min, 1 min) for 2 minutes in 0, 0.05, 0.1, 0.2 or 0.4 ml of DMEM supplemented with 10% FBS (A) or PBS (-) (B), and then replenished with 0.1 ml of fresh DMEM. After incubation for 48 hours, the viable cells were determined by MTT method, and expressed as a % of control cells not exposed to UV irradiation. Each value represents the mean of three determinations.

ascorbate (vitamin C) showed the most dramatic protective activity. Gallic acid was somewhat partially active, but NAC and catalase (data not shown) were totally inactive (Figure 7C), suggesting that the radical species produced by UV irradiation is not hydrogen peroxide.

Discussion

The present study demonstrates that DMEM is superior to α -MEM for culture of three normal oral cells HGF-1, HPC-1 and HPLF-1, based on its ability to maintain higher saturation density during one cycle of cell growth and during the aging process, despite the lack of potential to increase the total number of cell division during the life span (Figure

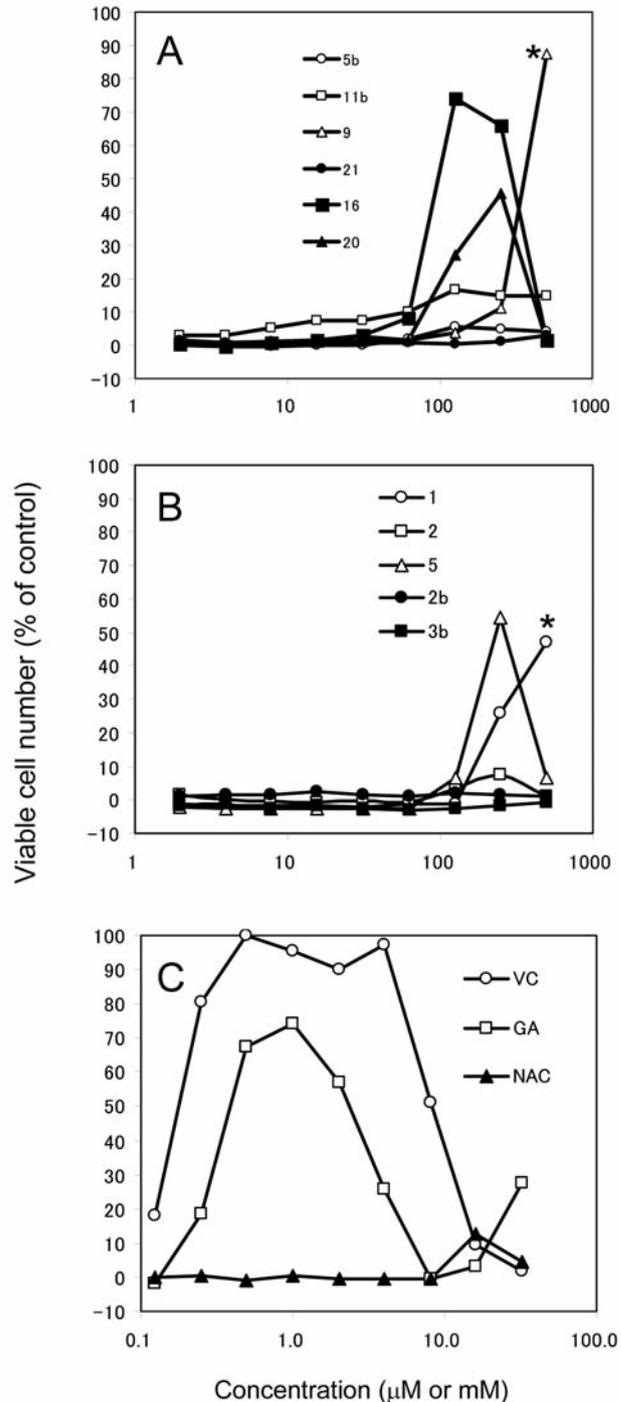


Figure 7. Protection of UV-induced cytotoxicity by group I and II compounds. HSC-2 cells were exposed to UV irradiation (6 J/m²/min, 1 min) in 0.2 ml of PBS(-) that contained different concentrations of group I compounds [5b, 11b, 9, 21, 16 or 20] (μM) (A), group II compounds [1, 2, 5, 2b, 3b] (μM) (B), or antioxidants (vitamin C, gallic acid, NAC) (mM) (C), and replaced by 0.1 ml of fresh medium (DMEM+10%FBS). After 24 hours, the viable cell number was determined by MTT method, and expressed as a % of control that has not been exposed to UV irradiation. *Precipitation of crystal. Each value represents the mean of three determinations.

2). During *in vitro* senescence, the BMI-1 protein level has been reported to decline, followed by the increase of p16 and p14, and the accumulation of p53 (23). This is supported by the present finding that p53 protein was expressed in all three aging normal oral cells (HGF-1, HPC-1, HPLF-1), regardless of the growing state (either logarithmically growing or resting), whereas the p53 protein level in OSCC cell lines (HSC-2) was much less (Figure 4). We were unable to detect the expression of BMI-1 protein in all six cells at any growth stage investigated (data not shown).

The present study has shown that group I compounds induced transient growth stimulation (hormesis) at early stages (within 24 hours) after treatment, confirming our similar results in normal skin and lung fibroblasts treated with [16] or [20] (22). It should be noted that the hormetic concentration range is so narrow that a slightly higher concentration induces cell death, suggesting that hormesis may be the mechanism by which the cells escape cell death. It has been recently reported that repeated mild heat stress induced wound healing in human skin fibroblast and enhanced the angiogenic ability of endothelial cells, suggesting the applicability of mild stress-induced hormesis for the modulation, intervention and prevention of aging (24). Mild loading of heat shock has been reported to reduce the initial mortality in a worm (25). Tai Chi exercise has been reported to stimulate endogenous antioxidant enzymes and reduce oxidative damage markers (26). These data suggest that the mild or moderate dose of treatment may provide the beneficial effect of hormesis, and possibly group I compounds with higher tumor-specificity may activate this hormetic response machinery.

We have established the simple method for the evaluation of the protective effect against UV irradiation, using this intact cell system. We found that sodium ascorbate and gallic acid, but not NAC nor catalase, protected the cells from UV-induced cellular damage, suggesting the involvement of radical species other than hydrogen peroxide and superoxide anion. Using this assay system, we found for the first time that both group I and II compounds protected cells from UV-induced cellular damage, suggesting their *in vivo* UV protective effect. Another by-product of the present study is our finding that all three normal oral cells (HGF-1, HPC-1, HPLF-1) were much more resistant to UV irradiation as compared with three human OSCC cell lines (HSC-2, HSC-3, HSC-4). Further studies are necessary to explain such a difference in UV sensitivity between normal and tumor cells.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No. 19592156).

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Received October 29, 2010

Revised April 12, 2010

Accepted April 20, 2010