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Hormetic and UV-Protective Effects of Azulene-related Compounds

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Abstract. *Background: We have previously reported a possible anti-inflammatory activity of azulene-, tropolone- and azulenequinone-related compounds. To further pursue the newly discoverd biological activity of these compounds, five compounds that inhibited nitric oxide production by activated macrophages were investigated for their possible hormetic and anti-radiation effects. Materials and Methods: Viable cell number of human oral normal cells (gingival fibroblast, pulp cell and periodontal ligament fibroblast) and three oral squamous cell carcinoma cell lines on treatment with various concentrations of each azulene-related compound was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Apoptosis induction was monitored by caspase-3 activation and DNA fragmentation. Results: Among five compounds, only benzo[b]cyclohepta[e][1,4]thiazine slightly stimulated the growth of all three normal cell types, but not tumor cell lines, at concentrations slightly higher than cytotoxic concentrations. Using a newly established evaluation system for UV-induced cellular damage, we found that this compound slightly but significantly protected the cells from UV-induced cellular injury, and its effect was synergistically enhanced by sodium ascorbate. Conclusion: These data suggest the possible application of benzo[b]cyclohepta[e][1,4]thiazine in UV protection therapy.*

Heterocylic compounds are known to display diverse biological activities (1). Azulene (2-5), an isomer of naphthalene, has a dipole moment and a resonance energy having intermediate values between that of benzene and naphthalene, and is considerably more reactive, when compared with two arenes. Azulene derivatives have been investigated for their synthesis and chemical reactions (6-8). They have been shown to have several biological activities, including antibacterial (9), anti-ulcer (10), and relaxant activity (11), inhibition of thromboxane A_2-induced vasoconstriction and thrombosis (12), acute toxicity and local anesthetic activity (13), and chemotherapeutic activity against mucous membrane diseases (14-15). However, the effects of azulene derivatives on cellular function have not yet been investigated in detail.

We 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-trichloroacethylazulene, 1,3-ditrichloroacetyl-4,6,8-trimethylazulene (16), 6,8,10-tribromobenzo[*b*]cyclohepta[*e*][1,4]oxazine, 6-bromo-2-chlorobenzo[*b*]cyclohepta[*e*][1,4]oxazine (17), 7-bromo-2-(4-hydroxyanilino)tropone and 2-(2-hydroxyanilino)-4-isopropyltropone (18) showed higher cytotoxicity against human oral squamous cell carcinoma (OSCC) cell lines as compared with normal human oral cells. We tentatively classified these highly tumor-specific compounds as Group I compounds. On the other hand, benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]**, 6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine **[2]** (19), benzo[*b*]cyclohept[*e*][1,4]oxazin-6(11*H*)-one **[5]** (20), 3-methyl-1-trichloroacetylazulene **[2b]** and 3-ethyl-1-trichloroacetylazulene **[3b]** (21) (Figure 1) inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like RAW264.7 cells. We tentatively classified these highly inhibitory substances for NO production as Group II compounds.

It has been reported that many toxic substances, environmental hormones, inorganic compounds, and even irradiation modulate the growth of cultured cells in a bi-phasic fashion, stimulating or inhibiting the growth of cultured cells at lower and higher concentrations, respectively. This growth-stimulating effect at lower concentrations is known as hormesis (22-23). 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 OSCC cell

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lines (24). We also reported that sodium fluoride failed to induce hormesis during the aging process of human oral normal cells, suggesting the occurrence of a hormetic response only at certain durations and concentrations (25). This was supported by our recently finding that 7-bromo-2-(4-hydroxyanilino)tropone and 2-(2-hydroxyanilino)-4-isopropyltropone 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) (26). We recently reported that Group I compounds slightly stimulated the growth of human periodontal ligament fibroblast (HPLF) cells only at restricted durations and concentrations, but did not affect that of human gingival fibroblast (HGF) and human pulp cells (HPC) cells, suggesting these compounds have minor hormetic effects (27). To confirm the generality of the occurrence of hormesis, we investigated whether five Group II compounds induce hormesis in human normal oral cells (HGF, HPC and HPLF) (27) and three human oral squamous cell carcinoma cell lines (HSC-2, HSC-4, Ca9-22) .

Using a newly established evaluation system for UV-induced cellular damage (27), we also investigated here whether Group 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) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO), dithiothreitol (DTT) (Wako Pure Chem. Ind., Osaka, Japan); sodium ascorbate (Tokyo Chemical Industry Co., Ltd., Tokyo), caspase-3 substrate (DEVD-*p*NA) (MBVL, Nagoya, Japan).

Synthesis of test 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(11*H*)-one **[5]**, 3-methyl-1-trichloroacetylazulene **[2b]**, 3-ethyl-1-trichloroacetylazulene **[3b]** were prepared as described previously (16-21).

Cell culture. HGF, HPC and HPLF cells were established from the first premolar extracted tooth in the lower jaw, and periodontal tissues of a twelve-year-old girl, as described previously (27). OSCC cell lines (HSC-2, HSC-4, Ca9-22) were cultured as described previously (16-18, 26).

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, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 ml of PBS (-) to minimize the evaporation of water from the culture medium. After 48 hours, the medium was removed by suction with an aspirator, and replaced with 0.1 ml of fresh medium containing different concentrations of single test compounds. The first well (500 μM, occasionally 250 or 25 μM) was diluted two-fold sequentially, with triplicate wells for each concentration. Cells

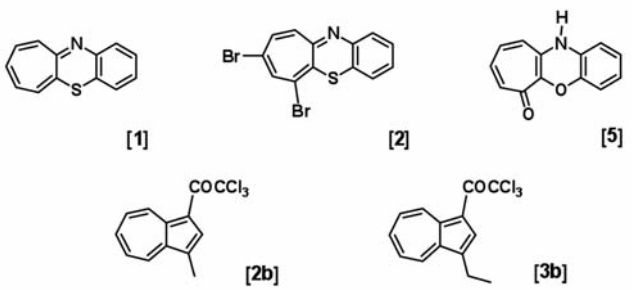

Figure 1. *Chemical structure of Group II compounds: benzo[b]cyclohepta[e][1,4]thiazine **[1]**, 6,8-dibromobenzo[b]cyclohepta[e][1,4]thiazine **[2]**, benzo[b]cyclohepta[e][1,4]oxazin-6(11H)-one **[5]**, 3-methyl-1-trichloroacetylazulene **[2b]**, 3-ethyl-1-trichloroacetylazulene **[3b]**.*

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 Labsystem, Helsinki, Finland).

Assay for hormesis. The hormetic response was evaluated by the maximum response in each dose-response curve, as described previously (22-23, 26).

Assay for UV protection. The medium of cells attached to 96-microwell plates was replaced with DMEM+10% FBS or PBS(–). The cells were added with various concentrations of azulenes, and then immediately placed at 21 cm distance from a UV lamp (wavelength: 253.7 nm) and exposed to UV irradiation (6 J/m^2/min) for different durations (0-2 min) (27). The media were replaced with fresh DMEM+10% FBS and cells again cultured at 37˚C in a CO_2 incubator until 48 hours after the start of irradiation.

Assay for DNA fragmentation. The cells (6×10^4) were seeded on 5-cm dishes and incubated for 48 hours to allow complete attachment. After changing the medium, cells were exposed to UV irradiation for 30-240 s, and incubated for a further 6 hours. After washing twice with PBS(–), cells were collected by scraping with a rubber policeman on ice and spun down in an eppendorf tube. Cells were lysed with 50 μl lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate]. The solution was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 2 hours at 50°C and then mixed with 50 μl NaI solution [40 mM Tris-HCl (pH 8.0), 7.6 mM NaI, 20 mM EDTA-2Na] followed by 250 μl of ethanol. After centrifugation for 20 minutes at 20,000×*g*, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA-2Na]. Each sample (10-20 μl, equivalent to 5×10^5 cells) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed as described previously (28). DNA from HL-60 cells induced to apoptosis by UV irradiation (6 J/m^2/min, 1 min) (29) was run in parallel as positive control.

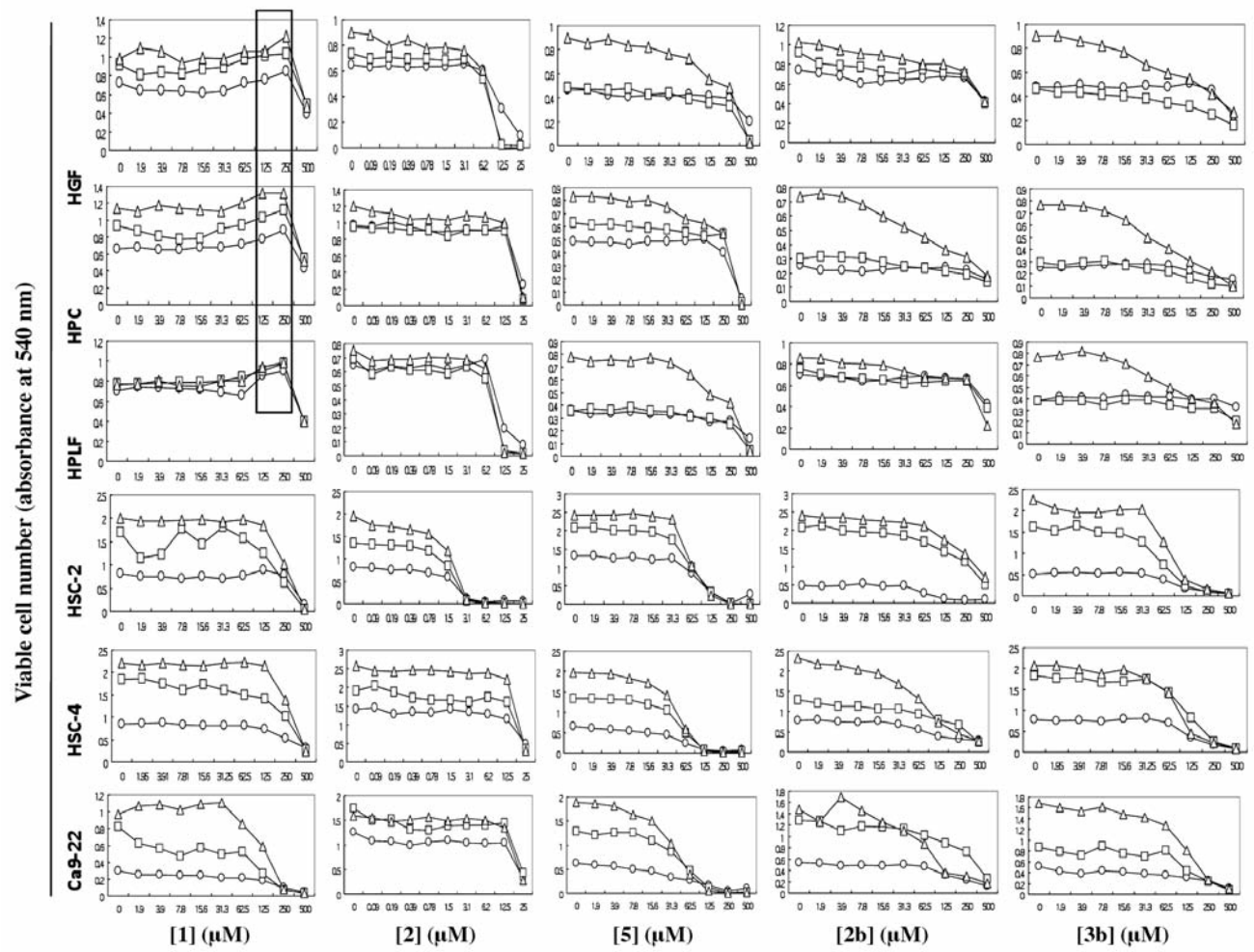

Figure 2. *Hormetic effects of Group II compounds. HGF (23 population doubling level (PDL)), HPC (24 PDL), HPLF (24 PDL), HSC-2, HSC-4 and Ca9-22 cells were treated for 24 (○), 48 (□) or 72 (△) hours with* ***[1]****,* ***[2]****,* ***[5]****,* ***[2b]*** *or* ***[3b]****, and the viable cell number was determined by MTT method. Each value represents the mean of three determinations. The hormetic effect shown by* ***[1]*** *is highlighted in the box.*

Assay for caspase-3 activation. The untreated cells (8×10^4) were seeded on 50-mm dishes, and incubated for 72 hours to allow complete adherence. Cells were then incubated for a further 6 hours in fresh medium containing the indicated concentrations of samples. Cells were washed twice with PBS(−) and lysed with 150 μl of lysis solution (50 mM Tris-HCl, pH 7.5, 0.3% NP-40, 1 mM DTT). Cells were collected by scraping with a rubber policeman and transferred to an eppendorf tube. After standing the tube for 10 min on ice and centrifugation for 5 min at 10,000×*g*, the supernatant was collected. Lysate (50 μl, equivalent to 100 μg protein) was mixed with 50 μl lysis solution containing substrates for caspase-3 (DEVD-*p*NA). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by microplate reader as described previously (28).

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

Results

Hormetic response. Among 5 compounds tested, only **[1]** stimulated the growth stimulatory activity against three human oral normal cells (HGF, HPC, HPLF), but not against three oral squamous cell carcinoma cell lines (HSC-2, HSC-4, Ca9-22) (Figure 2, Table I). This hormetic effect was observed only at concentrations of 125-250 μM, slightly higher than the cytotoxic concentration (500 μM) (Figure 2). Furthermore, the maximum hormetic response induced by **[1]** was only 24.6, 21.9 or 22.2% in HGF, HPC and HPLF, respectively. The incidence of hormesis did not depend on the incubation time (24, 48 or 72 hours). The remaining compounds induced hormesis to much lesser extents (Figure 2).

Table I. *Hormetic response of human oral normal and tumor cells after treatment with 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]**.*

	Incubation time	Maximum hermetic response (%)					
		Normal cells			Tumor cells		
Compd.	(h)	HGF	HPC	HPLF	HSC-2	HSC-4	Ca9-22
[1]	24	6.4-12	6.5-21.9	2.5-19.9	0-10.6	0-4.6	0
	48	7.7-11.5	5.5-19.1	5.0-20.7	6.9-8.6	0	0
	72	7.4-24.6	13.6-17.7	1.6-22.2	0	0.3-2.4	3.7-13.7
[2]	24	0-0.03	2.9-4.8	3.9-15.5	0	0-4	0
	48	0	0	0	0	0.12	0
	72	0	0	0	0	0	0
[5]	24	0	1.4-2.0	0	0-1.3	0	0
	48	0	0-0.7	0-2.9	0-2.6	0-0.7	0
	72	0-4.5	0	0	0	0	0-5.3
[2b]	24	0-2.5	0	0-2.0	0-8.2	0	0-5.7
	48	0-2.9	0-1.5	0	0-2.5	0	0
	72	0-1.3	0-2.2	0	0-2.7	0	0-14.6
[3b]	24	3.2-5.2	2.9-5.0	4.1-9.7	4.1-9.7	0-3.3	0
	48	0	0-1.1	0-0.9	0-2.3	0-1.1	0-3.0
	72	0-8.0	0.5-1.9	0-5.2	0	0-0.6	0

These values were calculated from data shown in Figure 2.

Table II. *Cytotoxicity of Group II compounds against human oral normal and tumor cells.*

	Incubation time	CC_{50}(μM)						
		Normal cells			Tumor cells			
Compd.	(h)	HGF	HPC	HPLF	HSC-2	HSC-4	Ca9-22	TS
[1]	24	6.518-532	556-597	525-541	137-342	187-322	109-303	1.7-3.7
	48	516-605	325-533	437-586	153-208	137-281	92-153	2.1-4.3
	72	494-565	513-218	271-594	139-327	167-284	154-174	1.7-3.5
[2]	24	8.8-12	9.1-21	11-24	2.1-17	3.1-21	3.8-20	1.0-3.2
	48	4.8-8.3	4.5-19	8.7-25	1.9-12	2.2-19	2.2-20	1.0-2.9
	72	5.0-7.8	4.2-18	8.8-23	1.9-8.3	2.3-19	2.4-19	1.1-2.7
[5]	24	340-350	369-411	232-400	42-84	14-29	18-40	7.6-13
	48	383-383	195-358	270-287	8.0-61	7.9-53	23-49	6.3-22
	72	247-267	125-311	208-279	13-59	10-47	20-36	6.1-22
[2b]	24	548-636	501-616	564-579	83-124	114-151	67-133	2.9-6.1
	48	229-526	303-453	333-510	31-51	60-79	79-93	5.1-6.1
	72	376-515	133-171	385-443	38-71	52-83	36-80	4.8-7.2
[3b]	24	431-475	328-773	670-957	111-115	106-113	111-211	4.4-5.0
	48	283-389	137-156	534-653	59-174	113-129	66-126	2.4-4.6
	72	193-228	62-136	107-204	74-87	86-130	73-127	1.6-1.6

TS: Tumor specificity. Each value represents experimental data from two experiments which were carried out in triplicate.

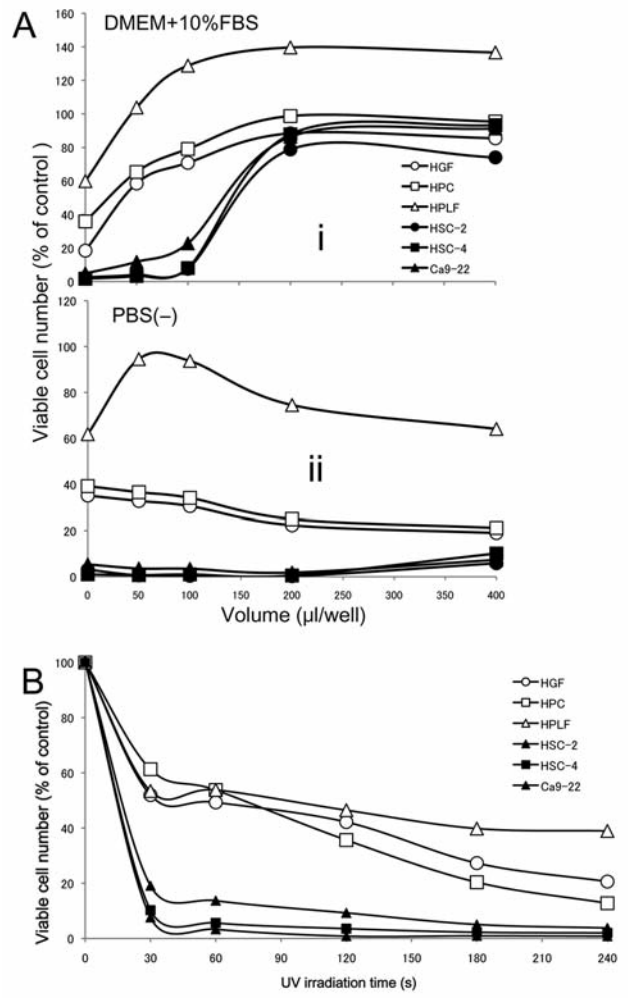

Figure 3. *Effect of UV irradiation on the viability of cultured oral cells. HGF (15 PDL), HPC (16 PDL), HPLF (15 PDL), HSC-2, HSC-4 and Ca9-22 cells were A: exposed to UV irradiation (6 J/m^2/min, 1 minute) for 2 min in 0, 0.05, 0.1, 0.2 or 0.4 ml of DMEM supplemented with 10% FBS (i), or PBS (−) (ii), or B: exposed for 30, 60, 120, 180 or 240 s in 0.1 ml PBS (−). After removing the medium or PBS (−), the cells were incubated for 48 hours in fresh culture medium. The viable cell number was then determined by MTT method, and expressed as a percentage that of control cells not exposed to UV irradiation. Each value represents the mean of three determinations.*

Tumor specificity. We compared the cytotoxic activity of five azulene compounds against both normal and tumor cells to delineate the tumor specificity (TS), defined as the ratio of the mean CC_{50} value for normal cells to the mean CC_{50} value for tumor cells. Among these five compounds, compound **[5]** showed the highest tumor specificity (TS=6.3-22), followed by **[2b]** (TS=2.9-7.2) > **[3b]** (TS=1.6-5.0)> **[1]** (TS=1.7-4.3)>**[2]** (1.0-3.2) (Table II). This indicates that the extent of hormesis does not relate to tumor specificity.

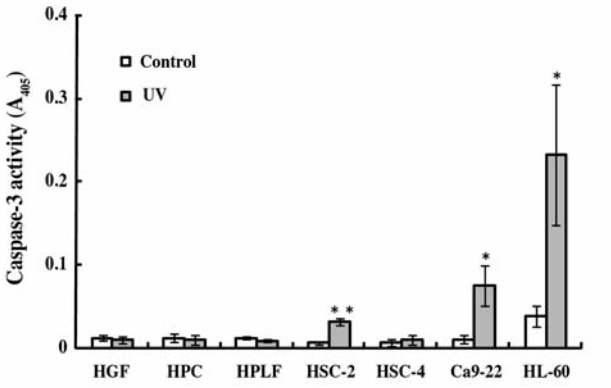

Figure 4. *Effect of UV irradiation on caspase-3 activity. HGF (15 PDL), HPC (16 PDL), HPLF (15 PDL), HSC-2, HSC-4, Ca9-22, and HL-60 cells were unexposed (white bars) or exposed (gray bars) to UV irradiation (6 J/m^2/min, 1 min) for 1 min in PBS (−). After removing the PBS(−), the cells were incubated for 6 hours in fresh culture medium. The cells were lysed for determination of caspase-3 activity. Each value represents the mean±S.D. of triplicate assays. $^*p<0.05$, $^{**}p<0.01$ relative to the control.*

UV sensitivity of normal and tumor cells. UV sensitivity of normal and tumor cells was first compared. The cytotoxic effect of UV irradiation on OSCC cell lines was diminished by increasing the volume of culture medium, preventing direct contact of the monolayer cultures with the circulating air (Figure 3Ai). When the culture medium was replaced by PBS(−), such a protective effect disappeared. This suggests the presence of radical scavenger(s) or UV-absorbing substance(s) in the culture medium (Figure 3Ai). Normal cells were much more resistant to UV irradiation, as compared with OSCC cell lines, both in culture medium and PBS(−) (Figure 3A). The viability of OSCC (HSC-2, HSC-4 and Ca9-22) was reduced to 7.6, 10.3 and 19.1% upon exposure to short (30 s) UV irradiation. On the other hand, normal oral cells (HGF, HPC, HPLF) maintained much higher viability (20.6, 12.8 and 39.0%) even when they were exposed to much longer UV irradiation (240 s) (Figure 3B).

Type of cell death induced by UV irradiation. UV irradiation induced caspase-3 activity slightly but significantly ($p<0.01$ or 0.05) in HSC-2, HSC-4 and Ca9-22 cells, but to much lesser extent than that induced in HL-60 cells. On the other hand, UV irradiation did not enhance, but rather slightly inhibited caspase-3 activity in normal cells (HGF, HPC, HPLF) (Figure 4). The caspase-3 activity was slightly increased at 4 hours after irradiation (Figure 4). It was unexpected that UV irradiation did not induce inter-nucleosomal DNA fragmentation in both normal and tumor cells (Figure 5), in contrast to HL-60 cells where a typical DNA laddering pattern was observed. It should be noted that

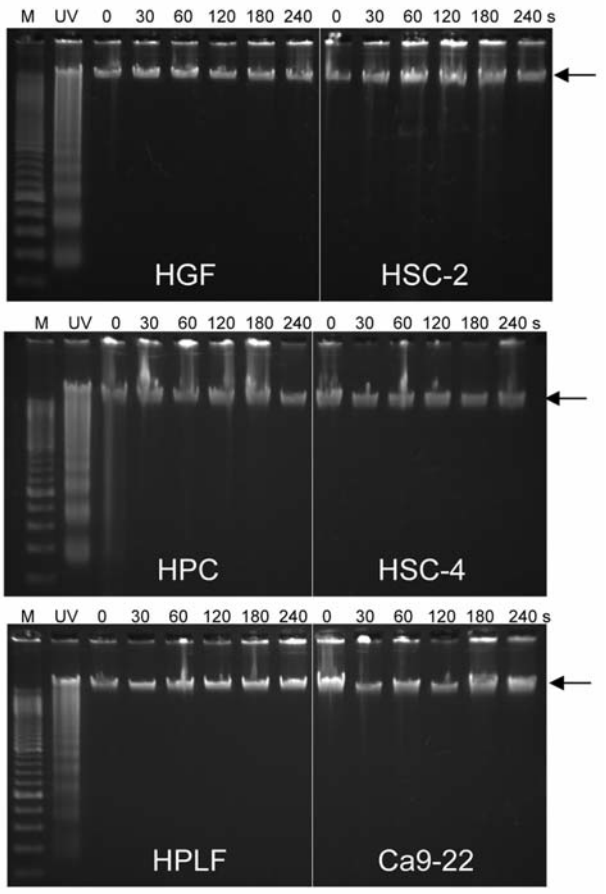

Figure 5. *Effect of UV irradiation on induction of DNA fragmentation. HGF (15 PDL), HPC (16 PDL), HPLF (15 PDL), HSC-2, HSC-4, Ca9-22, and HL-60 cells were exposed to UV irradiation (6 J/m^2/min) for 0, 30, 60, 120, 180 or 240 s in 0.1 ml medium. The cells were incubated for a further 6 hours in the same culture medium, and lysed for the assay of DNA fragmentation by agarose gel electrophoresis. UV, DNA from UV-irradiated HL-60 cells.*

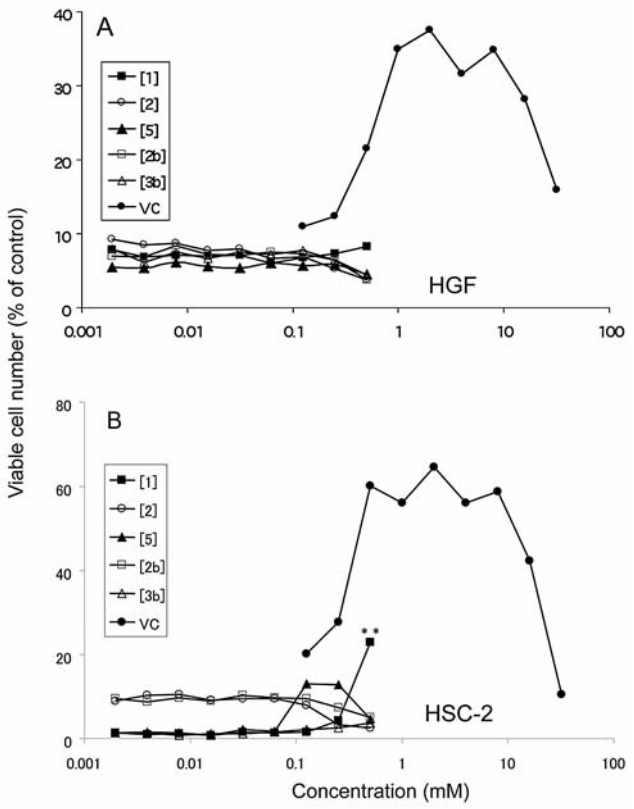

Figure 6. *Protective effect of azulenes and sodium ascorbate (VC) on UV-induced cytotoxicity. HGF (A) and HSC-2 (B) cells were placed in PBS(−) containing different concentrations of azulenes [1, 2, 5, 2b, 3b] or sodium ascorbate (VC), and then exposed to UV irradiation (6 J/m^2/min) for 4 min and 30 s, respectively. After removal of PBS(−), the cells were further incubated for 48 hours in fresh culture medium. The viable cell number was then determined by MTT method, and expressed as a percentage of the viable cell number of unirradiated cells. Each value represents the mean from triplicate assay. ** $p<0.001$ compared to UV treated control cells without compound treatment.*

UV irradiation induced a large DNA fragment (indicated by arrows in Figure 5). However, the biological significance of this phenomenon is unclear.

Protective effect of azulene on UV-induced cytotoxicity. We next investigated the protective effects of increasing concentrations of azulene compounds on UV-induced cellular damage in HGF and HSC-2 cells, using sodium ascorbate (vitamin C, 0.5-5 mM) as a positive control (Figure 6). We found similar trends or both HGF and HSC-2 cells, however, the difference of the sensitivity between compounds were more pronounced in HSC-2 cells. Only **[1]** among five azulenes slightly, but significantly ($p<0.001$) showed a UV-protective effect at the highest concentration (0.5 mM) (indicated by arrows). Due to the water-insolubility, addition of higher concentrations of **[1]** could not be achieved.

We found that combination of **[1]** (500 μM) and vitamin C (0.2 mM) showed significantly higher UV-protective effect than the latter alone ($p<0.05$), and exceeded that expected for the summation of these two, indicating the synergistic UV-protective effect of **[1]** and vitamin C (Figure 7).

Discussion

The present study demonstrated that only **[1]** among five azulene compounds induced a hormetic response at near cytotoxic concentration at different time points. This suggests that the hormetic response depends more on the concentration, rather than the treatment time. Similaly, sodium fluoride (25), three herbal extracts (24), gefitinib (30), 2-aminotropone derivatives (26) and CO_2 laser irradiation (31) showed hormetic effects at concentrations

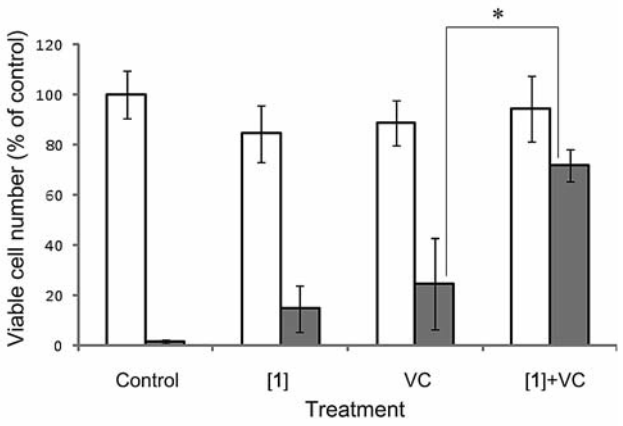

Figure 7. *Synergistic protective effect of* ***[1]*** *and sodium ascorbate (VC). HSC-2 cells were unexposed (white bars) or exposed (shaded bars) to UV irradiation (6 J/m^2/min) for 1 min in PBS(−) without (control) or with* ***[1]*** *(250 μg/ml), or VC (0.2 mM), or a combination of both* ***[1]*** *(250 μg/ml) and VC (0.2 mM). After removing the PBS(−), the cells were incubated for a further 24 hours in fresh culture medium to determine the viable cell number by MTT method. Each value represents the mean±S.D. of triplicate samples. $^*p<0.05$.*

slightly higher than cytotoxic concentrations in human oral cells. This suggests that hormesis may be responsible for the mechanism by which cells escape from the cell death signal. Taking this and previous studies (24-27, 31) together the human oral normal cells (HGF, HPC, HPLF) and oral squamous cell carcinoma cell lines (HSC-2, HSC-4, Ca9-22) studied here showed marginal maximum hormetic responses (maximum response was usually below 20%), in contrast to previous reports (22-23). This suggests that the extent of the hormetic response may depend on the type of cell, rather than the type of compound.

The present study also demonstrated that only **[1]** among five compounds slightly rescued cells from UV-induced cytotoxicity, confirming our recent report (27). This further suggests the possible link of hormesis induction and UV protection by **[1]**. Due to the limitation imposed by the water-solubility of **[1]**, we were unable to apply much higher (millimolar) concentrations of **[1]**. Instead, we found for the first time that compound **[1]** synergistically enhanced the suboptimal UV protective effect of vitamin C, by an as yet to be identified mechanism, in HSC-2 cells, suggesting that the points of action of **[1]** and of vitamin C may be different. We have observed a similar UV-protective effect of **[1]** in human gingival fibroblast, but the protective effect of **[1]** was much less, as compared with that observed in HSC-2 cells (data not shown). The present simple UV protective assay may be applicable to identify more active compounds. For this assay, more water-soluble materials are recommended.

We found that compound **[5]** showed the highest tumor-specificity by an as yet to be unidentified mechanism. The order of potency of these five azulene compounds in inducing hormesis was not the same as that for tumor specificity, suggesting that the mechanism of the induction of these phenomena may be different.

In conclusion, the present study demonstrates that **[1]** not only induced hormetic growth-stimulating activity in three normal oral cells, but also protected the cells from UV-induced injury. These data suggest the possible application of **[1]** in UV-protection therapy. UV-irradiation results in several types of DNA damage. In response to such DNA damage, mammalian cells exert DNA damage responses including cell cycle check points, DNA repair, apoptosis and premature senescence (32-33). It remains to be investigated what the point of action of **[1]** together with vitamin C is.

Acknowledgements

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