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Tumor-specificity and Type of Cell Death Induced by Trihaloacetylazulenes in Human Tumor Cell Lines

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Abstract. *Twenty trihaloacetylazulene derivatives with one atom of fluorine, chlorine, bromine or iodine was investigated for their tumor-specific cytotoxicity and apoptosis-inducing activity against three human normal cells (gingival fibroblast, HGF; pulp cell, HPC; periodontal ligament fibroblast, HPLF) and four human tumor cell lines (squamous cell carcinoma, HSC-2, HSC-3, HSC-4; promyelocytic leukemia, HL-60). There was no apparent difference in the cytotoxic activity between 2-methoxyazulenes **[1a-1e, 2a-2e]** and 2-ethoxyazulenes **[3a-3e, 4a-4e]**. Trichloroacetylazulenes **[2a-2e, 4a-4e]** generally showed higher cytotoxicity and tumor-specificity (expressed as a TS value) as compared with the corresponding trifluoroacetylazulenes **[1a-1e, 3a-3e]**. Substitution of chloride **[1c, 2c, 3c. 4c]**, bromide **[1d, 2d, 3d, 4d]** or iodine **[1e, 2e, 3e, 4e]** at the C-3 position further enhanced cytotoxic activity against four tumor cell lines, especially HL-60 cells. Among twenty trihaloacetylazulene derivatives, two compounds **[2d]** and **[4c]** showed the highest tumor specificity (TS=>3.5 and >2.5, respectively). Compounds **[2d]** and **[4c]** induced apoptotic cell death characterized by caspase-3, -8 and -9 activation and internucleosomal DNA fragmentation in HL-60 cells. On the other hand, compounds **[2d]** and **[4c]** induced autophagic cell death characterized by lower activation of caspases, lack of DNA fragmentation, vacuolization and autophagosome formation detected by acridine orange and LC3-GFP fluorescence, without the decline of the intracellular concentration of three major polyamines in HSC-4 cells. The cytotoxic activity of **[4c]**, but not **[2d]**, was slightly reduced by 3-methyladenine, an inhibitor of autophagy. These results suggest the diversity of cell death type induced in human tumor cell lines by trihaloacetylazulene derivatives.*

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Azulene (1-4), an isomer to naphthalene, has a dipole moment and a resonance energy with intermediate values between that of benzene and naphthalene, and is considerably more reactive, when compared with the two arenes. Azulene derivatives have been investigated in the past for their synthesis and chemical reactions (5-7). Their derivatives have shown several biological activities, including antibacterial activity (8), anti-ulcer activity (9), relaxant activity (10), inhibition of thromboxane A_2-induced vasoconstriction and thrombosis (11), acute toxicity and local anesthetic activity (12), and chemotherapeutic activity against mucous membrane diseases (13, 14). We have recently shown the quantitative structure-activity relationship (QSAR) of a total of 81 azulene (15, 16), tropolone (17, 18) and azulenequinone (19, 20) derivatives. We found that 1,3-difluoroazulene (15), 2,4-dibromo-7-methoxytropone (17), 3-morpholino-1,5-azulenequinone and 3,7-dibromo-1,5-azulenequinone (19) inhibited the NO production by activated mouse macrophages, without (15) or with (17, 19) the inhibition of iNOS mRNA and protein expression. We also found that 2-acetylaminoazulene, diethyl 2-chloroazulene-1,3-dicarboxylate and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate showed higher tumor-specific cytotoxicity than the parent compounds, such as azulene and guaiazulene (16). Tropolone derivatives with phenolic OH group, hinokithiol, its tosylate and methyl ethers showed relatively higher tumor-specific cytotoxic activity (18). 3-(3-Guaiazulenyl)-1,5-azulenequinone and 7-isopropyl-3-

(4-methylanilino)-2-methyl-1,5-azulenequinone showed relatively higher tumor-specific cytotoxicity and induced apoptosis in human tumor cell lines, possibly *via* the activation of both mitochondria-independent (extrinsic) and -dependent (intrinsic) pathways (20).

It has been reported that hexafluorotrihydroxyvitamin D_3 derivatives show higher differentiation-inducing activity (21, 22) than the parent compound (21, 22). We have also recently reported that thirteen trichloroacetylazulenes showed higher tumor-specific cytotoxic activity than the corresponding thirteen trifluoroacetylazulenes (23). These results suggest that the introduction of halogen may enhance the biological activity of the parent compound. However, it is still not clear whether the introduction of other halogens shows a similar enhancing effect. In this study, therefore, twenty trihaloacetylazulene derivatives with one atom of fluorine, chlorine, bromine or iodine (Figure 1) were investigated for their tumor-specific cytotoxicity and apoptosis-inducing activity against three human normal cells (gingival fibroblast, HGF; pulp cell, HPC; periodontal ligament fibroblast, HPLF) and four human tumor cell lines (oral squamous cell carcinoma, HSC-2, HSC-3, HSC-4; promyelocytic leukemia, HL-60).

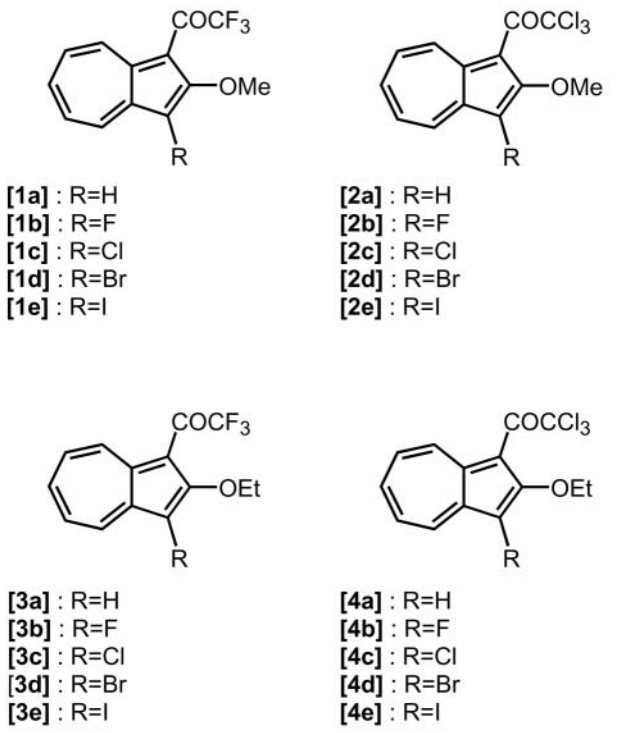

Figure 1. *Structure of the ten trifluoroacetylazulenes **[1a-1e, 3a-3e]** and ten trichloroacetylazulenes **[2a-2e, 4a-4e]**.*

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbeccos's modified Eagle's medium (DMEM), RPMI 1640 (Sigma Chem. Co., St. Louis, MO, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA) (Wako Pure Chem, Ind, Ltd, Osaka, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., St. Louis, MO, USA).

Synthesis of trihaloacetylazulenes. Trihaloacetylazulene derivatives were synthesized, according to methods previously published: 1-trifluoroacetyl-2-methoxyazulene **[1a]** (24-27), 1-trifluoroacetyl-3-fluoro-2-methoxyazulene **[1b]** (25, 28-30), 1-trifluoroacetyl-3-chloro-2-methoxyazulene **[1c]** (25, 31-34), 1-trifluoroacetyl-3-bromo-2-methoxyazulene **[1d]** (25, 31-34), 1-trifluoroacetyl-3-iodo-2-methoxyazulene **[1e]** (25, 31, 32), 1-trichloroacetyl-2-methoxyazulene **[2a]** (24-27), 1-trichloroacetyl-3-fluoro-2-methoxyazulene **[2b]** (25, 28-30), 1-trichloroacetyl-3-chloro-2-methoxyazulene **[2c]** (25, 31-34), 1-trichloroacetyl-3-bromo-2-methoxyazulene **[2d]** (25, 31-34), 1-trichloroacetyl-3-iodo-2-methoxyazulene **[2e]** (25, 31, 32), 1-trifluoroacetyl-2-ethoxyazulene **[3a]** (24-27, 34), 1-trifluoroacetyl-2-ethoxy-3-fluoroazulene **[3b]** (25, 28-30), 1-trifluoroacetyl-3-chloro-2-ethoxyazulene **[3c]** (25, 31-34), 1-trifluoroacetyl-3-bromo-2-ethoxyazulene **[3d]** (25, 31-34), 1-trifluoroacetyl-2-ethoxy-3-iodoazulene **[3e]** (25, 31, 32), 1-trichloroacetyl-2-ethoxyazulene **[4a]** (24-27, 34), 1-trichloroacetyl-2-ethoxy-3-fluoroazulene **[4b]** (25, 28-30), 1-trichloroacetyl-3-chloro-2-ethoxyazulene **[4c]** (25, 31-34), 1-trichloroacetyl-3-bromo-2-ethoxyazulene **[4d]** (25, 31,-34), 1-trichloroacetyl-2-ethoxy-3-iodoazulene **[4e]** (25, 31, 32) (Figure 1).

Cell culture. Three human oral tumor cell lines (HSC-2, HSC-3, HSC-4) and three human normal cells (HGF, HPC, HPLF) were cultured in DMEM supplemented with 10% heat-inactivated FBS. Human promyelocytic leukemic HL-60 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. Tumor cell lines were obtained from the Riken Cell Bank. Normal cells were prepared from the periodontal tissues, according to the guidelines of the Institutional Board of Meikai University Ethic Committee (No. 0206) after obtaining the informed consent from the patients. Since HGF, HPC and HPLF cells have limited lifespan due to *in vitro* senescence (35), these cells were used at the population doubling level of 5-8 for the present study.

Assay for cytotoxic activity. Cells (other than HL-60 cells) were inoculated at $5x10^3$ cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 hours, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing various concentrations of test compounds. The first well contained 500 μM of the sample, and was diluted 2-fold sequentially, with three replicate wells for each concentration. Cells were incubated for another 24 hours, and the relative viable cell number was then determined by the MTT method. In brief, cells were washed with phosphate-buffered saline without calcium and magnesium (PBS(-)), replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another four hours. The cells were 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) (16, 18, 20). A_{540} of the control cells was usually in the range from 0.40 to 0.90.

Table I. *Cytotoxic activity of trihaloacetylazulenes.*

		Cytotoxic activity (CC_{50}:μM)							
		Normal human cells			Human tumor cell lines				
Compd.	MW.	HGF	HPLF	HPC	HSC-2	HSC-3	HSC-4	HL-60	TS
[1a]	254.2	>500.0	>500.0	>500.0	449.0	>481.0	>478.6	>293.1	><1.2
[1b]	272.2	>500.0	>500.0	>500.0	>500.0	496.9	>500.0	>432.5	><1.0
[1c]	288.7	>166.7	>166.7	>166.7	150.1	147.2	>166.7	84.5	><1.2
[1d]	333.1	>500.0	>498.1	>500.0	>500.0	453.6	>500.0	79.4	><1.3
[1e]	380.1	>500.0	>500.0	>500.0	>412.7	308.0	>500.0	56.5	><1.6
[2a]	303.6	>437.9	>442.1	382.5	281.6	331.3	331.5	53.8	>1.7
[2b]	321.6	>466.6	398.6	390.9	288.8	249.4	254.7	35.8	>2.0
[2c]	338.0	192.3	264.2	259.1	199.2	148.8	58.9	<4.3	>2.3
[2d]	382.5	>175.8	>228.3	191.2	97.7	82.8	38.3	6.5	>3.5
[2e]	429.5	125.1	>152.0	>144.6	101.9	124.8	96.6	16.9	>1.7
[3a]	268.3	>495.6	>500.0	>500.0	>452.4	>500.0	>500.0	>426.2	><1.1
[3b]	286.2	>500.0	>500.0	>500.0	325.8	>489.9	>500.0	290.9	><1.2
[3c]	302.7	>500.0	>500.0	>500.0	>291.2	>392.4	>442.4	112.7	><1.6
[3d]	347.1	>166.7	>166.7	>166.7	>152.7	>166.7	>166.7	>152.4	><1.0
[3e]	394.1	>138.8	>166.7	>166.7	>135.9	>155.0	>166.7	86.1	><1.2
[4a]	317.6	>461.6	419.3	>457.9	214.2	273.6	339.4	71.3	>2.0
[4b]	335.6	390.0	363.5	386.2	223.3	244.8	440.4	30.0	1.6
[4c]	352.0	161.3	190.6	222.7	88.4	100.6	113.4	<4.2	>2.5
[4d]	396.5	92.8	79.2	150.0	57.1	94.3	66.2	<3.9	>1.9
[4e]	443.5	117.8	118.1	108.6	81.8	67.0	73.8	3.1	2.0

Each value represents mean from 3-6 independent experiments. MW: molecular weight; TS: tumor-specificity.

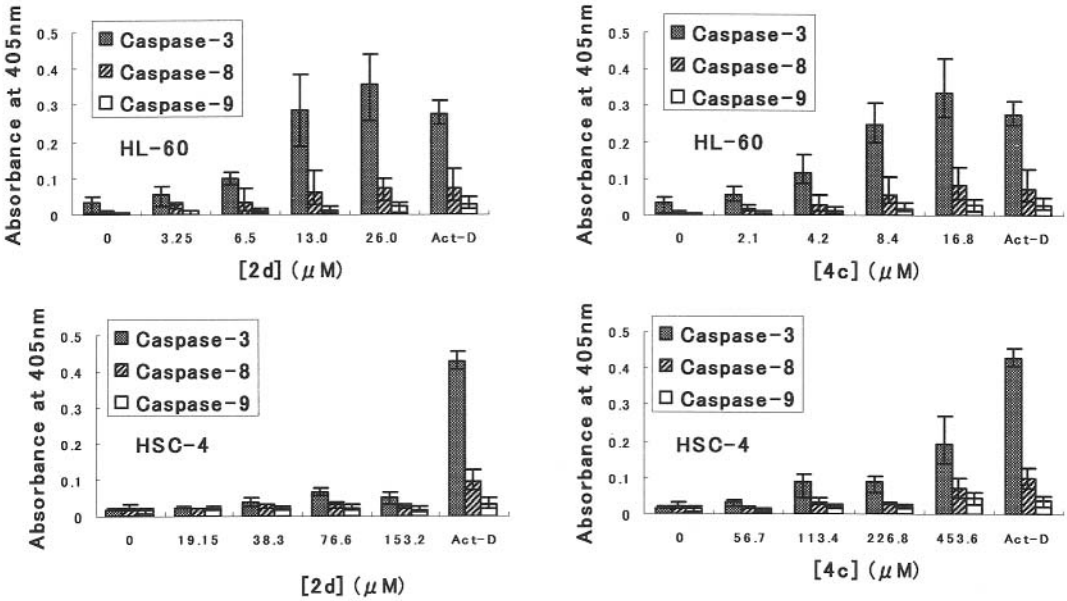

Figure 2. *Activation of caspases-3, -8 and -9 by two trichloroacetylazulenes* ***[2d, 4c]****. HL-60 and HSC-4 cells were incubated for 4 hours without (control) or with the indicated concentrations of* ***[2d]*** *or* ***[4c]*** *or 1 μg/mL actinomycin D (Act-D) (positive control). Each point represents mean±S.D. from 3-4 independent experiments.*

HL-60 cells were inoculated at $7.5x10^4$/0.1 mL in 96-microwell, and various concentrations of test compounds were added. After incubation for 24 hours, the viable cell number was determined by trypan blue exclusion, under a light microscope.

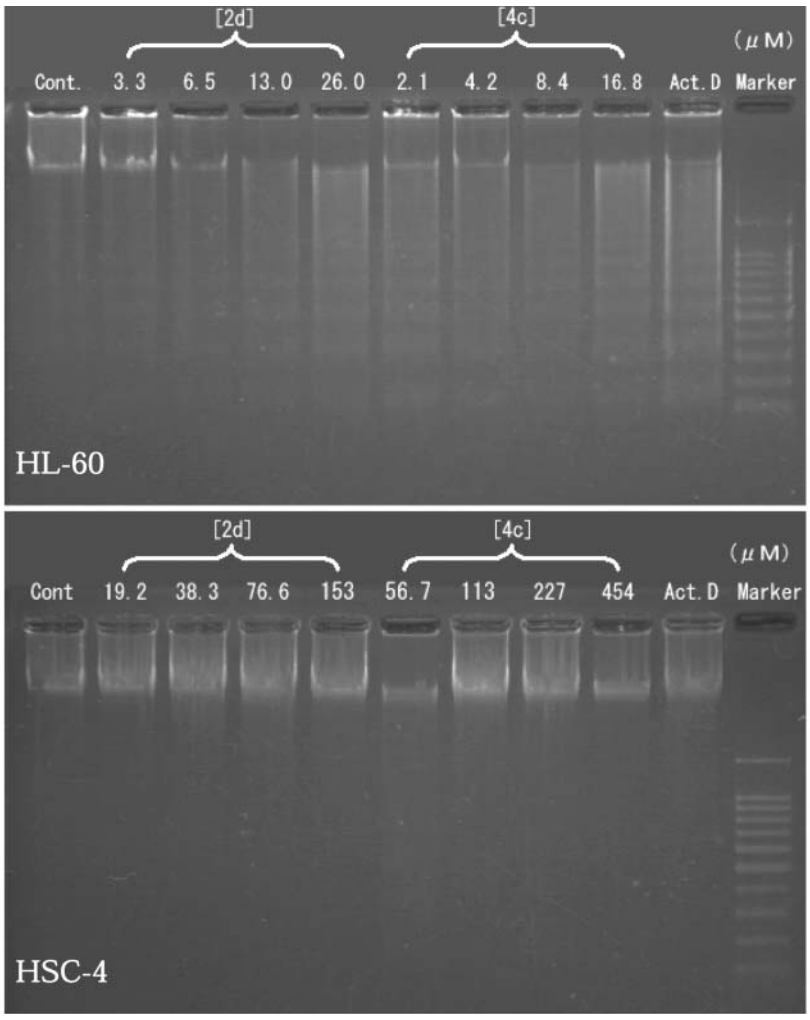

Figure 3. *Induction of DNA fragmentation by two trichloroacetylazulenes **[2d, 4c]**. Near confluent HL-60 (upper panel) or HSC-4 cells (low panel) were incubated for 6 hours with or without (cont) the indicated concentrations of **[2d]**, **[4c]** or 1 μg/mL actinomycin D (Act-D) (positive control). DNA was then extracted and applied to agarose gel electrophoresis. Marker DNA was run in lane 11. One representative example from three independent experiments is shown.*

The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Tumor-specificity (TS) was determined by the following equation: TS={[CC_{50} (HGF) + CC_{50} (HPC) + CC_{50} (HPLF)] / [CC_{50} (HSC-2) + CC_{50} (HSC-3) + CC_{50} (HSC-4) + CC_{50} (HL-60)]} x (4/3)

Assay for DNA fragmentation. Cells were washed once with PBS (-) and lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl sarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 hours at 50˚C, and then mixed with 50 μL NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and then 200 μL of ethanol. After centrifugation for 20 minutes at 20,000 xg, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Sample (10-20 μL) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by 1 μg/mL actinomycin D were run in parallel (16, 18, 20). After staining with ethidium bromide, DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc Inc, UVP).

Assay for caspase activation. Cells were washed with PBS(-) and lysed in lysis solution (MBL, Nagoya, Japan). After standing for ten minutes on ice and centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. Lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for two hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by plate reader (16, 18, 20).

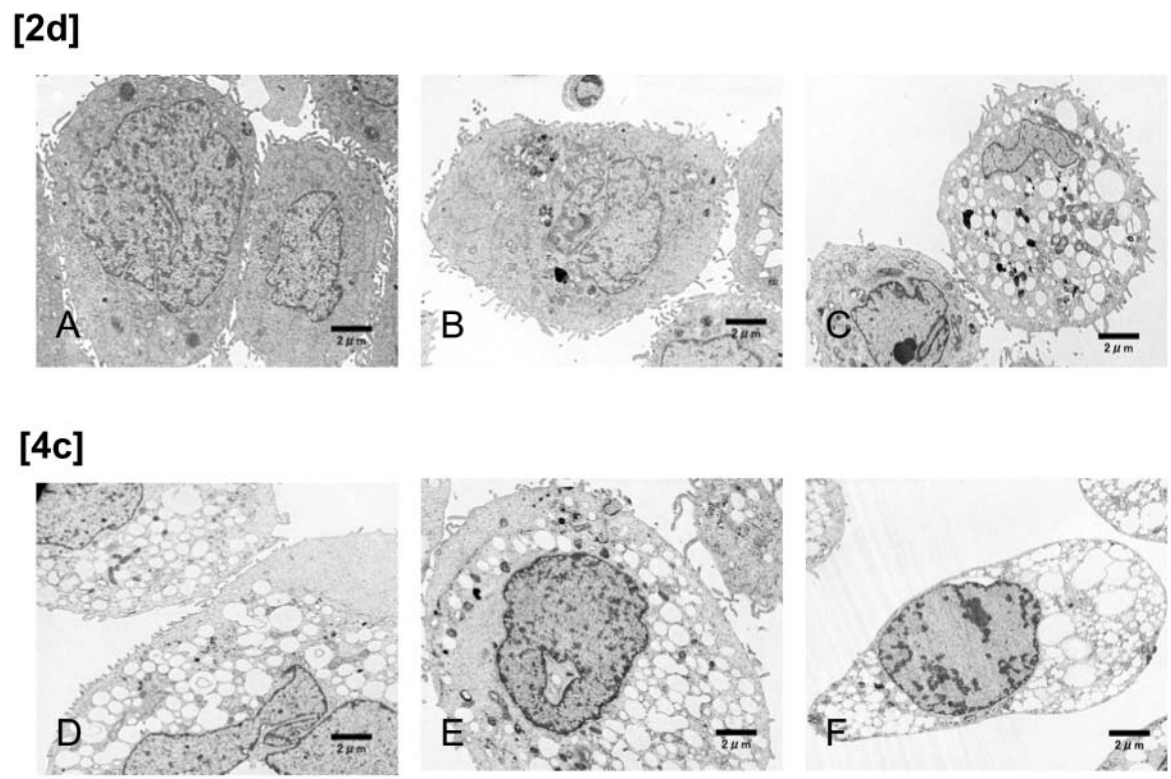

Figure 4. *Electron microscopy of control and dying HSC-4 cells. HSC-4 cells were incubated for 4 hours without (G) or with 38.3 (A), 76.6 (B) or 153.2 (C) μM **[2d]**, or 113.4 (D), 226.8 (E) or 435.6(F) μM **[4c]**, and then processed for the electron microscopy. Bar: 2 μm.*

Determination of polyamines. The cells were harvested by 0.25% trypsin-0.025%EDTA in PBS(-), washed twice with PBS(-), and extracted with 10% TCA. After centrifugation for five minutes at 10,000 xg, the deproteinized supernatant was collected and stored at –40˚C. The polyamines in the supernatant were determined by HPLC, after Dansyl-derivatization. Cadaverin-2HCl was used as internal standard (IS). A modified procedure of dansylation was performed according to a previously described method (36). Then 50 μL of the supernatant (or of the standard solution), 10 μL internal standard solution, 50 μL of saturated Na_2CO_3 solution and 100 μL of dansyl chloride (DNS) solution (10 mg/mL acetone) were added. After being vortexed, the mixing was continued for one hour at room temperature. Excess DNS was neutralized by reaction with proline (150 mg/mL). The dansylated derivatives were extracted into 0.2 mL of toluene. The extract was evaporated in airflow, and the residue was dissolved in 200 μL 80% CH_3CN/H_2O, and 10 μL was injected into a HPLC system. HPLC separation was performed with a Column SHISEIDO CAPCELL PAK C_{18} (4.6 mm I.D., 35 mm in length). The mobile phase for elution was a linear gradient between 50% acetonitrile in water (mobile phase A) and 80% acetontrile in water (mobile phase B), the time program was described below (0-5 min, A 100%; 5-13 min, A 100 %-0 %; 13-20 min, A 0%; 20-25 min, A 100%) at a flow rate at 0.7 mL/min. Fluorescence intensity was detected at excitation at 337 nm, and emission at 521 nm.

Construction of plasmids. cDNA encoding LC3 was obtained by RT-PCR from the total RNA of HSC-2 cells with the LC3 sense primer (5’-GGGAATTCATGCCGTCGGAGAAGACCTT-3’) and LC3

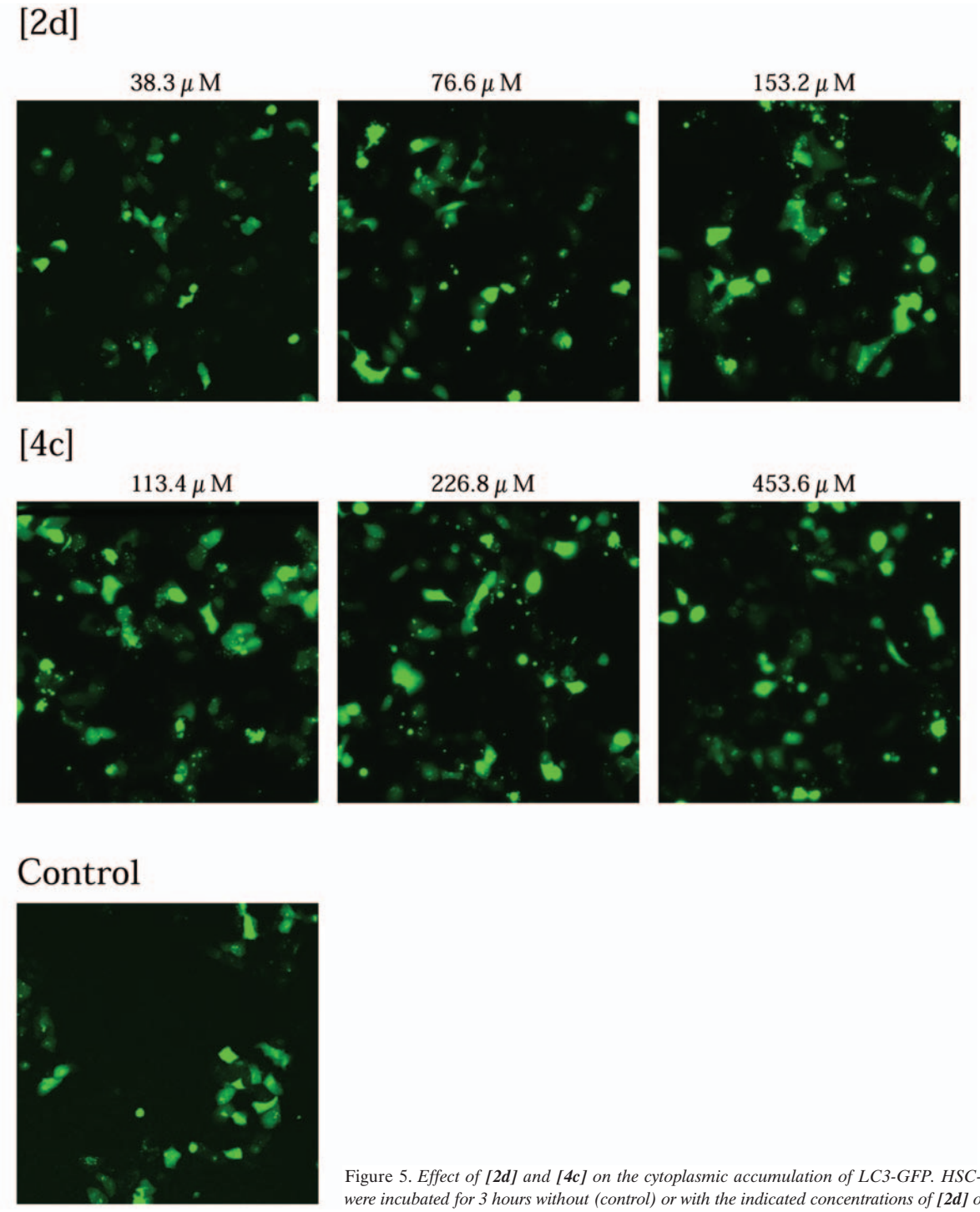

Figure 5. *Effect of **[2d]** and **[4c]** on the cytoplasmic accumulation of LC3-GFP. HSC-4 cells were incubated for 3 hours without (control) or with the indicated concentrations of **[2d]** or **[4c]**.*

antisense primer (5'-GGGAATTCTAGATTACACTGACAATTT CATCC-3'). It was subcloned into the *Eco*RI site of pAcGFP1-C2, a GFP fusion protein expression vector (Clontech Laboratories, Inc., Mountain View, CA, USA). The plasmid constructs was verified by DNA sequencing using the Applied Biosystems 310 DNA sequencer.

Transfection. HSC-4 cells were seeded at $0.5x10^6$ cells/well in a 24 well plate, and the next day, the cells were transfected with a mixture of 1.5 μg of plasmid DNA and 0.7 μl of Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA, USA). After transfection for 18 hours, the cells were used for each experiment.

Assay for LC3 accumulation to the autophagosome. GFP-LC3 transfected HSC-4 cells were observed by a Laser Scanning Microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany), using the following filters: excitation filter 488 nm, emission filter 505-530 nm.

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co.) as described previously (37, 38). HSC-4 cells were stained with 1 μg/mL acridine orange for 15 minutes. Samples were then examined under a laser scanning microscope LSM510, using the following filter: excitation filter 488 nm, emission filter 505-530 nm and >650 nm.

Electron microscopy. The cells were harvested by 0.25% trypsin-0.025%EDTA in PBS(-), and pelleted by centrifugation at 1,000 rpm for five minutes. The cells were fixed for one hour with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, postfixed for one hour with 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated, then embedded in Araldite 502 (CIBA-GEIGY, Basel, Swiss; NISSHIN EN Co. Ltd., Tokyo, Japan). Fine sections were stained with uranyl acetate and lead citrate, and then observed under a JEM-1210 electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 100 KV (39).

Results

Structure and activity relationship. There was no apparent difference in the cytotoxic activity between the 2-methoxyazulenes **[1a-1e, 2a-2e]** and the 2-ethoxyazulenes **[3a-3e, 4a-4e]** (Table I). The trichloroacetylazulenes **[2a-2e, 4a-4e]** generally showed higher cytotoxicity and tumor-specificity (expressed as a TS value) as compared with the corresponding trifluoroacetylazulenes **[1a-1e, 3a-3e]**. In particular, the trifluoroacetylazulenes **[1a, 1b, 1d, 1e, 3a, 3b]** showed much lower cytotoxic activity whereas 1-trichloroacetyl-3-bromo-2-ethoxyazulene **[4d]** and 1-trichloroacetyl-2-ethoxy-3-iodoazulene **[4e]** showed the highest cytotoxicity for both normal and tumor cells (Table I). In general, trichloroacetylazulenes have shown higher cytotoxicity than tropolone (18) and azulenequinones (20). Trifluoroace-tylazulenes have shown generally lower cytotoxic activity than the corresponding azulenes (16). Substitution of chloride **[1c, 2c, 3c. 4c]**, bromide **[1d, 2d, 3d, 4d]** or iodine **[1e, 2e, 3e, 4e]** at the C-3 position further enhanced their cytotoxicity activity against four tumor cell lines especially the HL-60 cells. Among the twenty compounds, compounds **[2d]** and **[4c]** showed the highest tumor specificity (TS=>3.5 and >2.5, respectively) (Table I).

Apoptosis induction in HL-60 cells. Compounds **[2d]** and **[4c]** induced caspase-3, -8 and -9 activation (Figure 2) and internucleosomal DNA fragmentation (Figure 3) in HL-60 cells. On the other hand, these compounds induced these apoptosis markers less efficiently in HSC-4 cells (Figures 2, 3).

Induction of autophagic cell death in HSC-4 cells. Transmission electron microscopy showed that compounds **[2d]** and **[4c]** induced vacuolization, without affecting the cell surface microvilli and nuclear membrane except at the highest concentration in HSC-4 cells (Figure 4). Compounds **[2d]** and **[4c]** induced the translocation of the LC3-GFP fusion protein into autophagosome (Figure 5). However, transient expression of LC3-GFP may cause severe cellular damage at higher concentration. Therefore, the possibility of autophagy induction by another method, a labeling of acidic vesicular organelles, such as autophagosomes, by acridine orange (37, 38) was tested. Compounds **[2d]** and **[4c]** induced the granular distribution of acridine orange in the autophagosome of HSC-4 cells, further supporting the occurrence of autophagy. It is interesting to note that there was an optimal concentration of acridine staining, since the acridine staining intensity declined at higher concentration (Figure 6). The cytotoxic activity of **[4c]**, but not **[2d]**, was slightly reduced by 3-methyladenine, an inhibitor of autophagy (Figure 7).

We have previously reported that the intracellular concentration of putrescine declined during the apoptotic cell death of HL-60 cells (40, 41). Therefore, we investigated whether compounds **[2d]** and **[4c]** induce similar change in the putrescine level. These compounds failed to induce the decline of putrescine and other polyamines (spermidine, spermine) in HSC-4 cells (Figure 8).

Discussion

The present study demonstrated that all ten trichloroacetylazulenes show higher cytotoxic activity against tumor cell lines than the corresponding ten trifluoroacetylazulenes, confirming our previous finding (23). The cytotoxic activity was significantly enhanced by the addition of a chlorine, bromine or iodine atom at the C-3 position. The HL-60 cells were found to be one order more sensitive to these compounds with extra halogen than the other oral squamous cell carcinoma cell lines, and the HL-60 cells were induced to undergo apoptosis by treatment with either compounds **[2d]** or **[4c]**. The activation of all caspase-3, -8 and -9 by these compounds suggests the activation of both mitochondria-independent (extrinsic) and -dependent (intrinsic) pathways (42). The extremely higher sensitivity of HL-60 cells to compounds **[2d]** and **[4c]** strongly suggests the applicability of these halogenated compounds for the treatment of leukemia cells, although further studying with various leukemic cell lines is necessary.

On the other hand, compounds **[2d]** or **[4c]** did not induce apoptotic markers so clearly in the HSC-4 cells. The much lower activation of caspases suggests the induction of non-apoptotic cell death (43). Compounds **[2d]** and **[4c]** induced vacuolization, and the granular distribution of acridine orange and translocation of the LC3-GFP into the autophagosome of

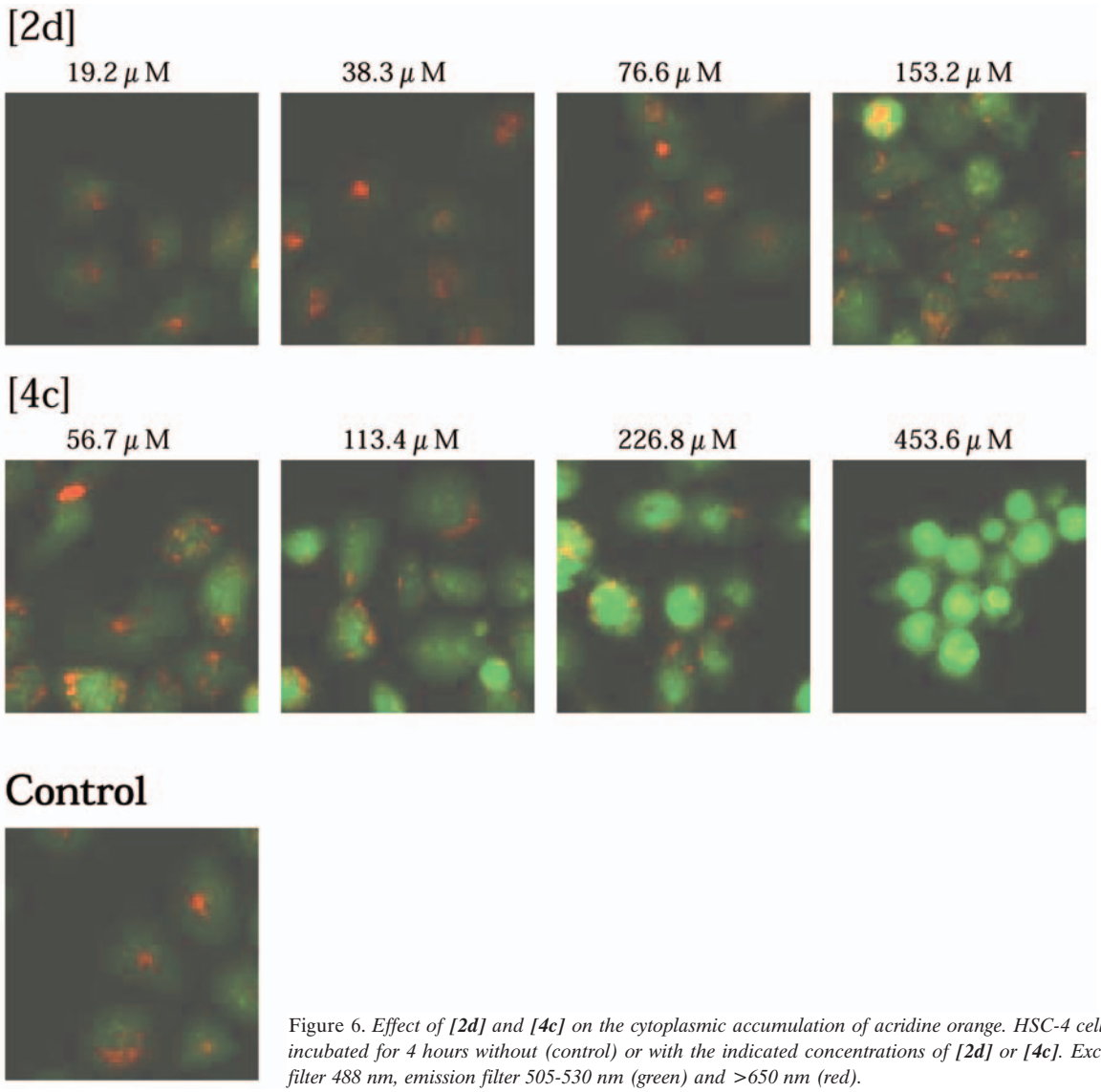

Figure 6. *Effect of **[2d]** and **[4c]** on the cytoplasmic accumulation of acridine orange. HSC-4 cells were incubated for 4 hours without (control) or with the indicated concentrations of **[2d]** or **[4c]**. Excitation filter 488 nm, emission filter 505-530 nm (green) and >650 nm (red).*

HSC-4 cells. The cell death induced by compound **[4c]**, but not **[2d]**, was slightly reduced by the addition of 3-methyladenine, an inhibitor of autophagy. This suggests the induction of autophagy in HSC-4 cells, at least to some extent.

The present study has demonstrated that both compounds **[2d]** or **[4c]** induced apoptotic cell death in HL-60 cells, whereas they induced autophagic cell death in HSC-4 cells. Since HSC-4 cells are much more resistant to these compounds, the induction of autophagic cell death may be coupled to the extent of drug resistance. This is supported by the induction of autography in malignant brain tumor cell lines, that are resistant to many of anticancer drugs (38, 44).

The intracellular level of putrescine did not decline during the autophagic cell death of the HSC-4 cells. This suggests that the decline of putrescine may be specific to apoptotic cell death, rather than to other types of cell death. More experiments are required to confirm this point.

In conclusion, it was found that the trihaloacetylazulene derivatives induced different type of cell death, depending on the type of cells. Further studying is required to clarify the action mechanisms by which such diversity is produced.

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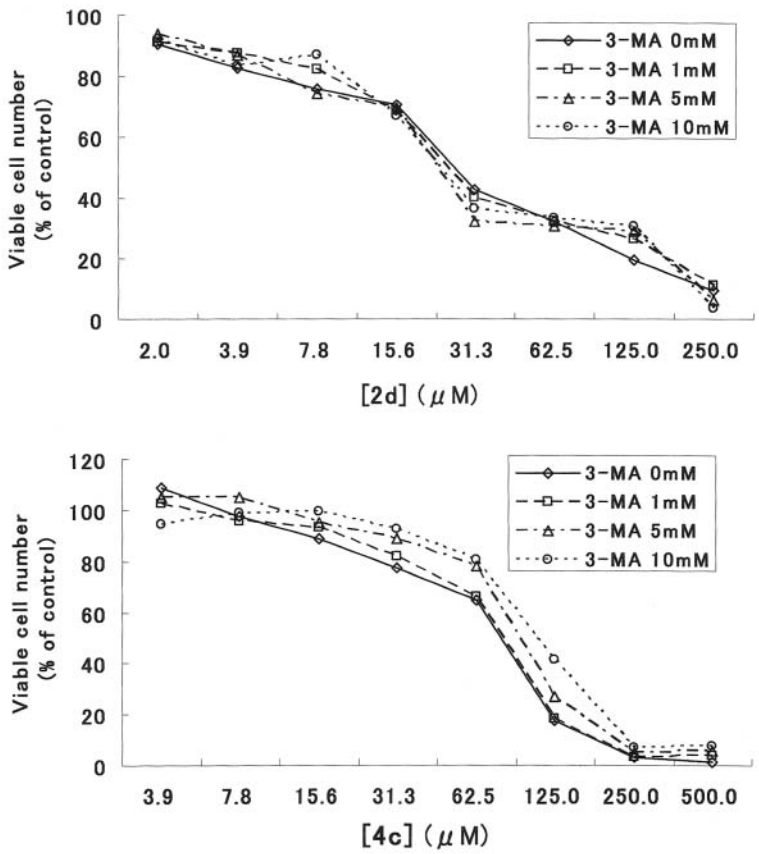

Figure 7. *Effect of 3-methyladenine on* ***[2d]*** *or* ***[4c]****-induced cytotoxicity. HSC-4 cells were incubated for 24 hours with the indicated concentrations of* ***[2d]*** *or* ***[4c]*** *in the presence of 0 (◇), 1 (□), 5 (△) or 10 (○) mM 3-methyladenine (3MA), and the viable cell number was determined by MTT method. Data was corrected for 3-methyladenine cytotoxicity. Each value represents mean from three determinants.*

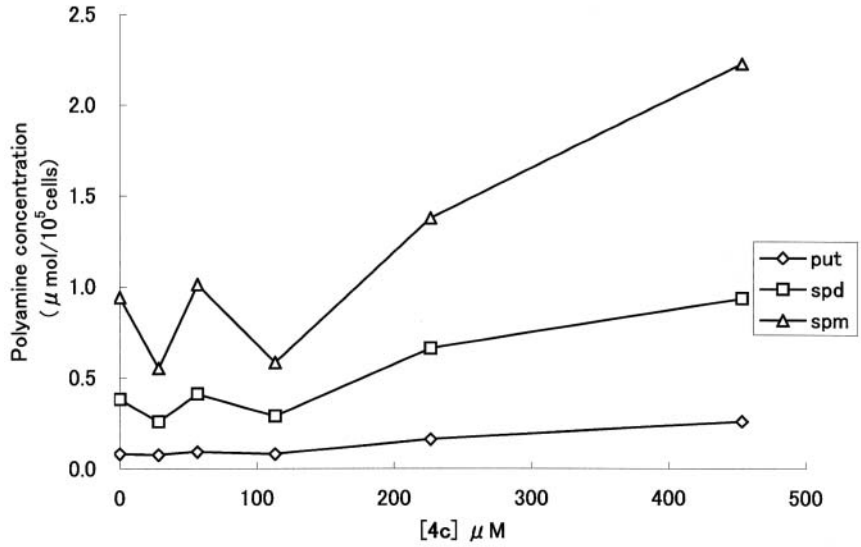

Figure 8. *Effect of* ***[4c]*** *on the intracellular concentration of polyamines. HSC-4 cells were incubated for 4 hours with the indicated concentrations of* ***[4c]****, and the intracellular concentration of polyamine was determined by HPLC. Each value represents the mean from six determinants. put: putrescine, spd: spermidine, spm: spermine.*

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