Cytotoxic Activity of Azulenes Against Human Oral Tumor Cell Lines

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Abstract. *We investigated 27 azulene derivatives for their relative cytotoxicity against three human normal cells and three human oral tumor cell lines. 2-Acetylaminoazulene **[4]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** showed higher tumor-specific cytotoxicity than azulene **[1]** and guaiazulene **[2]**. Four 1- and 3-halogenated compounds showed lower tumor specificity. The tumor-specific cytotoxic activity seems not to be related to the position of functional groups. All compounds showed no anti-HIV activity. Methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** induced apoptotic cell death (characterized by internucleosomal DNA fragmentation and caspase 3 activation) in HL-60 cells. ESR spectroscopy showed that methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** did not produce radical and less efficiently scavenged O_2^- (generated by HX-XOD reaction) and NO (generated from NOC-7). These data suggest that a radical-mediated oxidation mechanism may not be involved in the apoptosis induction by methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]**.*

Azulene **[1]** (structure shown in Figure 1) is an isomer to naphthalene, has a dipole moment and a resonance energy intermediate between that of benzene and naphthalene, but is considerably more reactive (1-4). Azulene derivatives have shown several biological activities, including antibacterial activity (5), anti-ulcer activity (6), relaxant activity (7), inhibition of thromboxane A_2-induced vasoconstriction and thrombosis (8), acute toxicity and local anesthetic activity (9). The accumulation of guaiazulene **[2]** at the mucous membrane suggested its application for chemotherapy of mucous membrane diseases (10, 11). However, the effects of azulene derivatives on cellular function have not been investigated in detail. We investigated here whether a total of 27 azulene derivatives display tumor-specific cytotoxic activity, using three normal human cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] and three human oral tumor cell lines [submandibular gland carcinoma (HSG) and oral squamous cell carcinoma (HSC-2, HSC-3)] and, if so, whether they induce apoptosis-associated characteristics (such as DNA fragmentation and caspase activation) in human tumor cells. We also investigated whether a radical-mediated mechanism is involved in the azulene-induced cytotoxicity, using ESR spectroscopy.

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Key Words: Azulenes, cytotoxic activity, apoptosis, caspase, DNA fragmentation.

Materials and Methods

Methods. The following chemicals and reagents were obtained from the indicated companies: Azulene **[1]**, guaiazulene **[2]** (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); Dulbeccos's modified Eagle medium (DMEM), RPMI 1640 (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS)(JRH, Bioscience, Lenexa, KS, USA); dimethyl sulfoxide (DMSO), diethylenetriaminepentaacetic acid (DETAPAC) (Wako Pure Chem, Ind, Ltd, Osaka, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), 3'-azido-2', 3'-

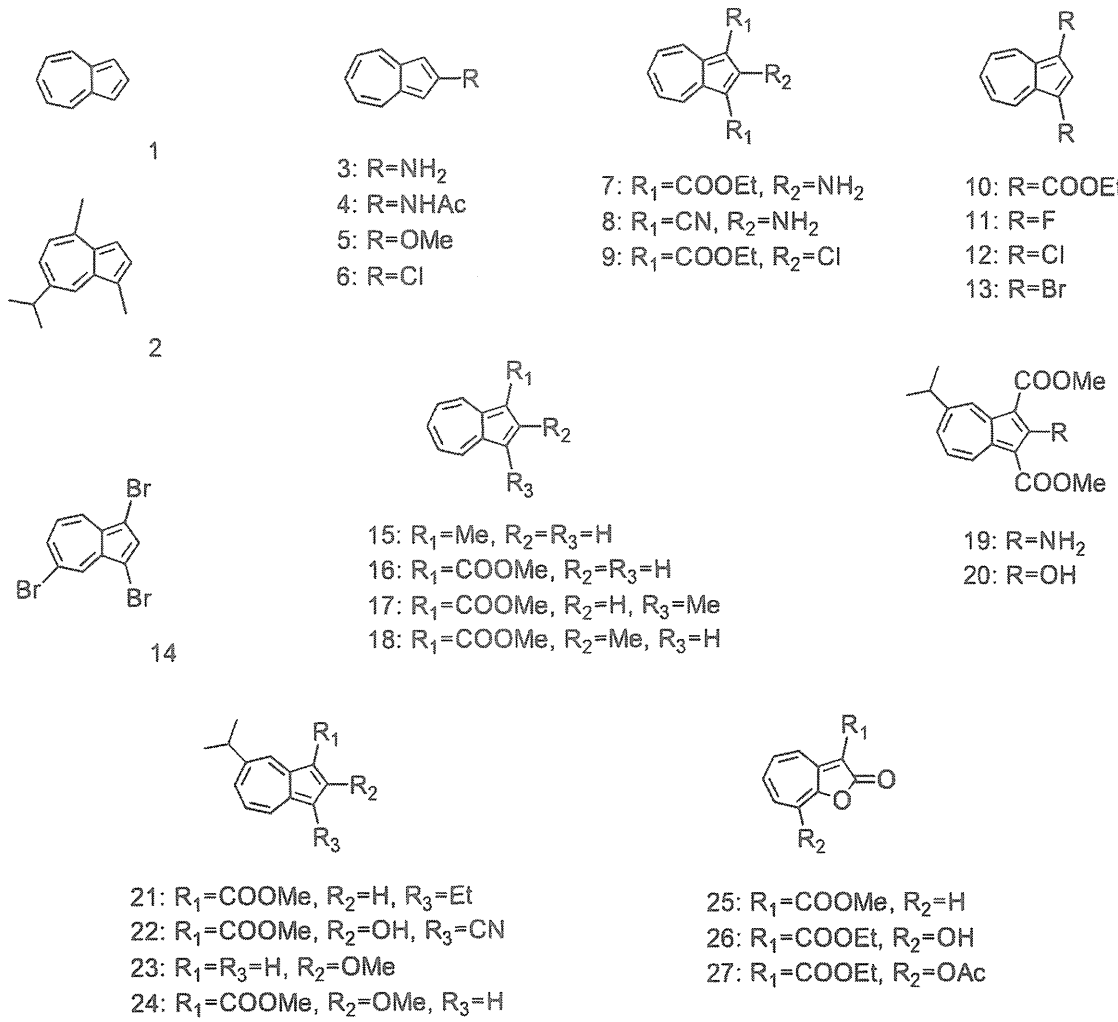

Figure 1. *Structure of azulene derivatives.*

dideoxythymidine (AZT), dideoxycytidine (ddC) (Sigma Chem Co., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD) from bovine erythrocytes, 1-hydroxyl-2-oxo-3-*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (Dojin, Kumamoto, Japan).

Synthesis of azulene derivatives. Azulene derivatives were synthesized, according to the published reports: Azulene **[1]** (12-14), guaiazulene **[2]** (12-14), 2-aminoazulene **[3]** (15-17), 2-acetylaminoazulene **[4]** (15-17), 2-methoxyazulene **[5]** (18, 19), 2-chloroazulene **[6]** (20), diethyl 2-aminoazulene-1,3-dicarboxylate **[7]** (15, 17, 19, 21), 2-amino-1,3-dicyanoazulene **[8]** (15, 17, 19, 21), diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** (20), diethyl azulene-1,3-dicarboxylate **[10]** (17), 1,3-difluoroazulene **[11]** (22), 1,3-dichloroazulene **[12]** (23, 24), 1,3-dibromoazulene **[13]** (25), 1,3,5-tribromoazulene **[14]** (24, 26), 1-methylazulene **[15]** (27-30), methyl azulene-1-carboxylate **[16]** (28, 29), methyl 3-methylazulene-1-carboxylate **[17]** (28, 29), methyl 2-methylazulene-1-carboxylate **[18]** (28, 29), dimethyl 2-amino-5-isopropylazulene-1,3-dicarboxylate **[19]** (15, 17, 19, 21), dimethyl 5-isopropyl-2-hydroxyazulene-1,3-dicarboxylate **[20]** (15, 17, 19, 21), methyl 3-ethyl-7-isopropylazulene-1-carboxylate **[21]** (28, 29), methyl 3-cyano-2-hydroxy-7-isopropylazulene-1-carboxylate **[22]** (15, 17, 19, 21), 5-isopropyl-2-methoxyazulene **[23]** (18, 19), methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** (18, 19), methyl 2*H*-cyclohepta[*b*]furan-2-one-3-carboxylate **[25]** (31), ethyl 8-hydroxy-2*H*-cyclohepta[*b*]furan-2-one-3-carboxylate **[26]** (32) and ethyl 8-acetoxycyclohepta[*b*]furan-2-one-3-carboxylate **[27]** (32).

Cell culture. Three human oral tumor cell lines (HSG, HSC-2, HSC-3) and three human normal cells [HGF (5-8 population doubling level (PDL)), HPC (5-8PDL), HPLF (5-8PDL)] were cultured in DMEM supplemented with 10% heat-inactivated FBS. Human promyelocytic leukemic HL-60 cells were cultured in RPMI 1640 supplemented with 10% FBS. Normal cells were prepared from periodontal tissues, according to the guideline of Meikai University Ethics Committee, after obtaining informed consent from the patients.

Table I. *Cytotoxic activity of azulenes.*

Compd.	Molecular Weight	Cytotoxic activity (CC_{50}: mM)						
		Normal human cells			Human tumor cell lines			
		HGF	HPC	HPLF	HSG	HSC-2	HSC-3	TS
1	128.17	2.38	2.52	2.29	2.50	1.33	2.33	1.2
2	198.31	0.40	0.19	0.19	0.11	0.16	0.12	2.0
3	143.19	>2.79	>2.79	>2.79	>2.79	>2.79	>2.79	>< 1.0
4	201.23	>1.99	>1.99	>1.99	0.34	0.34	0.98	>3.6
5	158.20	1.82	1.61	1.71	1.28	0.61	0.92	1.8
6	162.62	2.39	>2.46	1.86	>2.46	0.42	0.65	>< 1.9
7	287.32	0.98	0.81	0.92	>1.39	0.64	1.17	<0.8
8	193.21	>2.07	>2.02	>2.07	0.24	1.47	1.00	>2.3
9	307.76	1.22	>1.3	>1.3	0.18	0.24	0.25	>5.7
10	272.30	1.41	>1.47	1.43	1.17	1.30	>1.47	><1.1
11	164.16	0.91	1.01	0.90	0.79	0.62	0.58	1.4
12	197.06	1.78	1.77	1.85	>2.03	1.06	1.40	<1.2
13	285.97	0.27	0.26	0.30	0.31	0.19	0.15	1.3
14	364.86	0.42	0.32	0.43	0.41	0.17	0.30	1.3
15	142.20	0.55	0.59	0.54	0.60	0.58	0.51	1.0
16	186.20	0.76	0.43	0.45	0.25	0.45	0.35	1.6
17	200.24	0.41	0.36	0.40	0.22	0.25	0.49	1.2
18	200.24	0.39	0.38	0.38	0.22	0.14	0.35	1.6
19	301.34	0.95	0.85	0.95	1.07	0.28	0.80	1.3
20	302.33	0.95	0.65	0.78	0.87	0.43	0.38	1.4
21	256.35	0.35	0.30	0.30	0.16	0.18	0.29	1.5
22	269.30	0.96	1.12	0.84	0.89	0.84	0.78	1.2
23	200.14	0.48	0.40	0.43	0.22	0.46	0.23	1.4
24	258.32	0.18	0.43	0.17	0.11	0.11	0.11	2.4
25	204.19	>1.96	1.80	>1.96	0.83	1.42	1.68	>1.5
26	234.21	1.30	1.35	1.25	1.29	1.20	1.26	1.0
27	276.25	1.04	0.96	0.98	1.01	0.83	0.96	1.1

Assay for cytotoxic activity. Cells (other than HL-60 cells) were inoculated at 12 x 10^3 cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 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. Cells were incubated for another 24 hours and the relative viable cell number was then determined by MTT method. In brief, cells were replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another 4 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) (33). The A_{540} of control cells were usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Tumor specificity (TS) was determined by the following equation.

$$TS = \frac{[CC_{50}\,(HGF)] + [CC_{50}\,(HPC)] + [CC_{50}\,(HPLF)]}{[CC_{50}\,(HSG)] + [CC_{50}\,(HSC\text{-}2)] + [CC_{50}\,(HSC\text{-}3)]}$$

The viability of HL-60 cells was determined by trypan blue exclusion. HL-60 cells were inoculated at 5 x 10^4/0.1 mL in 96-microwell and various concentrations of test compounds were added. After incubation for 24 hours, the viable cell number was determined as described previously. The cell density of control cells at cell harvest was in the range of 8-9 x 10^5/ml.

Assay for DNA fragmentation. Cells were lysed with 50 μL of 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. After incubation, the lysate was mixed with 50 μL of NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0]. The lysate was mixed with 250 μL of ethanol and centrifuged 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 7.5). A 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 (Takara) and DNA from apoptotic

HL-60 cells induced by UV were used for calibration (34). The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay for caspase activation. Cells were washed with PBS and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice and centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. The 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 2 hours at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by plate reader.

Assay for radical intensity. The radical intensity of the test sample was determined at 25°C in 0.1 M Tris-HCl buffer (pH 7.4), 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9, 10) or in 0.1 M KOH (pH 12.5), using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 seconds; scanning time, 2 minutes. The radical intensity was defined as the ratio of peak height of these radicals to that of MnO (35).

To determine O_2^-, produced by HX-XOD reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, sample (in DMSO) 40 μL, H_2O or SOD 30 μL, XOD (0.5 U/mL in PB) 30 μL], the gain, time constant and scanning time were changed to 500, 0.1 seconds and 1 minute, respectively. The radical intensity was determined 1 minute after mixing. The O_2^- scavenging activity was expressed as SOD unit/mg sample, by calibration with the standard curve of SOD (35). The concentration required to reduce the radical intensity of DMPO-OOH by 50% (IC_{50}) was determined from the dose-response curve.

For the determination of NO radical, the sample was added to the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7 in 0.06 M phosphate buffer, pH 7.4. The gain and scanning time were changed to 250 and 2 minutes, respectively. The NO radical intensity was defined as the ratio of peak height of the 1st peak of carboxy-PTI, which was produced by the reaction of NO (derived from NOC-7) and carboxy-PTIO to that of MnO (35).

Assay for anti-human immunodeficiency virus (HIV) activity. MT-4 cells were infected with $HIV\text{-}1_{IIIB}$ at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock- infected (control) MT-4 cells (1.5 x 10^5/mL, 200 μL/well) were placed into 96-well microtiter plates and incubated in the presence of various concentrations of test samples. After incubation for 5 days at 37°C in a 5% CO_2 incubator, cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce MTT to a blue formazan product. The CC_{50} and 50% effective concentration (EC_{50}) were determined from the dose-response curve with mock-infected or HIV-infected cells, respectively (36). All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by selectivity index (SI), which was calculated by the following equation:

$$SI = CC_{50}/EC_{50}$$

Table II. *Anti-HIV activity of azulene.*

Compd.	CC_{50} (mM)	EC_{50} (mM)	SI
1	0.68	> 1.56	< 1
2	1.10	> 0.20	< 1
3	0.64	> 0.14	= 5
4	0.33	> 0.99	< 1
5	0.39	> 1.26	< 1
6	0.52	> 1.23	< 1
7	0.18	> 0.70	< 1
8	0.067	= 0.21	< 1
9	0.15	> 0.65	< 1
10	0.5	> 0.73	< 1
11	0.71	> 1.22	< 1
12	0.35	> 1.01	< 1
13	0.091	> 0.14	< 1
14	0.30	> 0.55	< 1
15	1.3	> 1.41	< 1
16	0.18	> 0.21	< 1
17	0.14	> 0.20	< 1
18	0.11	> 0.20	< 1
19	0.067	> 0.13	< 1
20	0.08	> 0.13	< 1
21	0.056	> 0.16	< 1
22	0.092	> 0.15	< 1
23	0.092	> 0.20	< 1
24	0.066	> 0.15	< 1
25	0.30	> 0.98	< 1
26	0.80	> 0.85	< 1
27	0.45	> 0.72	< 1
AZT	0.0657	0.000012	5617
ddC	4.859	0.00243	2002

Results

Structure and activity relationship. We first investigated 27 azulene derivatives for their relative cytotoxicity against three human normal cells (HGF, HPC, HPLF) and three human oral tumor cell lines (HSG, HSC-2, HSC-3) (Table I).

Azulene **[1]** showed the lowest cytotoxicity against both human tumor cells [CC_{50} (HSG)=2.50 mM; CC_{50} (HSC-2)=1.33 mM; CC_{50} (HSC-3)=2.33 mM] and normal human cells [CC_{50} (HGF)=2.38 mM; CC_{50} (HPC)=2.52 mM; CC_{50} (HPLF)=2.29 mM], yielding very weak tumor specificity (TS=1.2).

Guaiazulene **[2]** showed higher cytotoxicity against tumor cells [CC_{50} (HSG)=0.11 mM; CC_{50} (HSC-2)=0.16 mM; CC_{50} (HSC-3)=0.12 mM] and slightly lower cytotoxicity against normal cells [CC_{50} (HGF)=0.40 mM; CC_{50} (HPC)=0.19 mM; CC_{50} (HPLF)=0.19 mM], yielding a moderate magnitude of tumor specificity (TS=2.0). 2-Acetylaminoazulene **[4]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** and methyl 7-

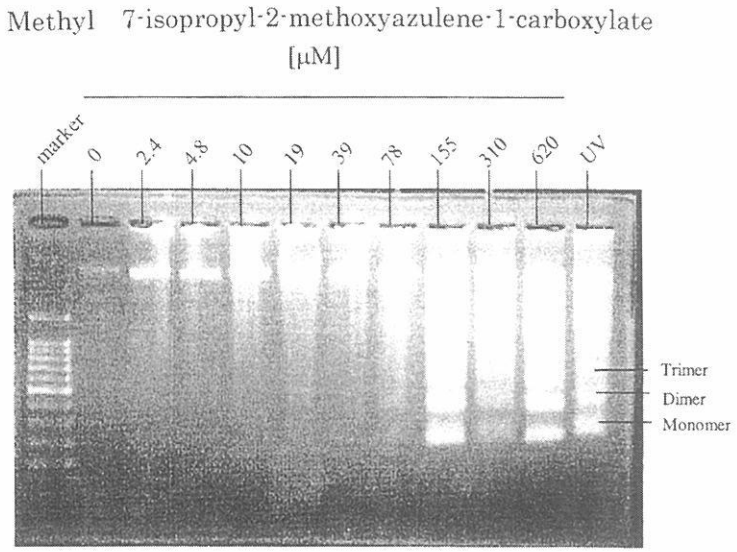

Figure 2. *Induction of DNA fragmentation by methyl 7-isopropyl-2-methoxyazulene-1-carboxylate* ***[24]*** *in HL-60 cells. HL-60 cells were inoculated at 5 x 10^5 cells/1 mL in 24-well plate, in fresh culture medium (RPMI1640 +10% FBS) with the indicated concentrations of methyl 7-isopropyl-2-methoxyazulene-1-carboxylate* ***[24]****. After incubation for 6 hours, DNA was extracted and applied to agarose gel electrophoresis. UV, DNA from apoptotic HL-60 cells induced by UV irradiation.*

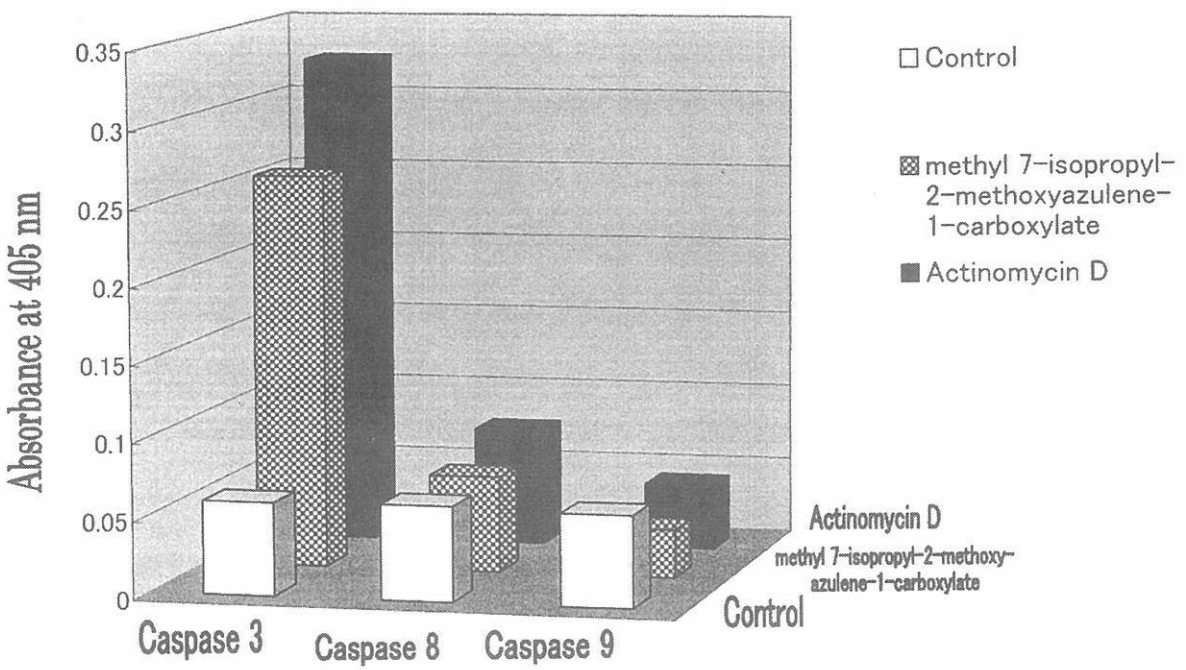

Figure 3. *Activation of caspase 3 by methyl 7-isopropyl-2-methoxyazulene-1-carboxylate* ***[24]*** *in HL-60 cells. HL-60 cells were incubated for 4 hours without (control) or with 155 μM methyl 7-isopropyl-2-methoxyazulene-1-carboxylate* ***[24]*** *or 1 μg/mL actinomycin D (positive control).*

isopropyl-2-methoxyazulene-1-carboxylate **[24]** showed comparable cytotoxic activity against tumor cells, but showed much lower cytotoxicity against normal cells, yielding the highest tumor-specific cytotoxicity (TS= >3.6, >5.7 and 2.4, respectively).

1,3-Dibromoazulene **[13]**, 1-methylazulene **[15]**, methyl 3-methylazulene-1-carboxylate **[17]**, methyl 2-methylazulene-1-carboxylate **[18]**, methyl 3-ethyl-7-isopropylazulene-1-carboxylate **[21]** and 5-isopropyl-2-methoxyazulene **[23]** were highly cytotoxic to both tumor and normal cells, yielding little or no tumor specificity (TS=1.3, 1.0, 1.2, 1.6, 1.5 and 1.4, respectively).

1- and 3-Halogenated compounds, such as 1,3-difluoroazulene **[11]**, 1,3-dichloroazulene **[12]**, 1,3-

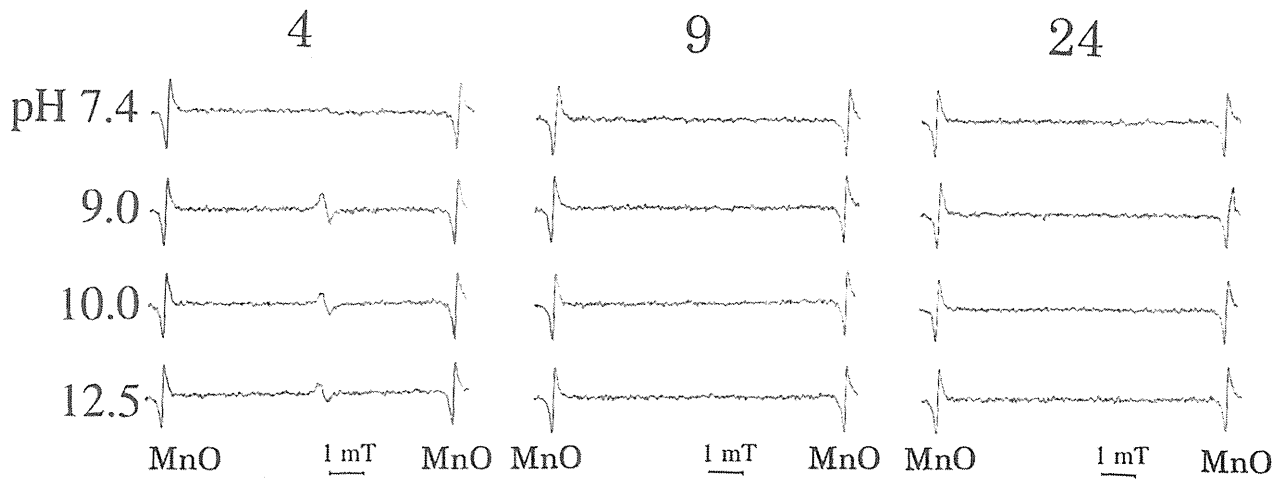

Figure 4. *ESR spectra of by 2-acetylaminoazulene [4], diethyl 2-chloroazulene-1,3-dicarboxylate [9] or methyl 7-isopropyl-2-methoxyazulene-1-carboxylate [24] (1 mg/mL) at increasing pH.*

dibromoazulene **[13]** and 1,3,5-tribromoazulene **[14]** showed lower tumor specificity (TS=1.4, <1.2, 1.3 and 1.3, respectively).

The tumor-specific cytotoxic activity seems not to be related to the position of functional groups, based on the following evidence: Although the aminoazulenes (2-acetylaminoazulene **[4]** and 2-amino-1,3-dicyanoazulene **[8]**) had relatively higher tumor specificity (TS= >3.6 and >2.3, respectively), the parent compound (2-aminoazulene **[3]**) and its diester derivatives (diethyl 2-aminoazulene-1,3-dicarboxylate **[7]**, dimethyl 2-amino-5-isopropylazulene-1,3-dicarboxylate **[19]**) showed much less tumor specificity (TS= ><1.0, <0.8 and 1.3, respectively). Two 2-methoxyazulene derivatives (5-isopropyl-2-methoxyazulene **[23]**, methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]**) showed considerably different tumor specificity to each other (TS=1.4 and 2.4, respectively). Two 2-chloroazulene derivatives, 2-chloroazulene **[6]** and diethyl 2-chloroazulene-1,3-dicarboxylate **[9]**, also showed considerably different tumor specificity to each other (TS=><1.9 and >5.7, respectively).

Most of the compounds, except for compound **[3]** (SI=5), showed no anti-HIV activity (SI<1), whereas two positive controls, such as AZT and ddC, showed potent anti-HIV activity (SI=5617 and 2002, respectively) (Table II).

Apoptosis induction. Since methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** showed higher cytotoxicity (CC_{50}=141 μM) against HL-60 cells than 2-acetylaminoazulene **[4]** (CC_{50}=720 μM) and diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** (CC_{50}=395 μM) (data not shown), we investigated whether methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** induced internucleosomal DNA fragmentation, a biochemical hallmark, in HL-60 cells. Figure 2 shows that this is the case. The optimal concentration was 0.155 mM. Methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** activated caspase 3 to a comparable extent as that attained by actinomycin D, without affecting caspase 8 and 9 activity (Figure 3). The apoptosis-inducing activity of 2-acetylaminoazulene **[4]** and diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** will be reported elsewhere.

Radical generation. 2-Acetylaminoazulene **[4]** produced radical under alkaline condition (pH 9.0-12.5), whereas diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** did not (Figure 4).

2-Acetylaminoazulene **[4]** efficiently scavenged O_2^- (generated by HX-XOD reaction) (IC_{50}=0.014 mM) (Figure 5). On the other hand, the O_2^- scavenging activity of methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** was very weak (IC_{50}= 1.61 mM). Diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** did not show O_2^- scavenging activity (IC_{50}>2.6 mM) (Figure 5).

2-Acetylaminoazulene **[4]** efficiently scavenged NO radical (generated from NOC-7) (IC_{50}=0.13 mM), whereas diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** were inactive (IC_{50}= >4.65 and >3.90 mM, respectively) (Figure 6).

Discussion

We found that, among 27 azulene derivatives, 2-acetylaminoazulene **[4]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** showed higher tumor-specific cytotoxic activity, as compared with azulene **[1]** and guaiazulene **[2]**.

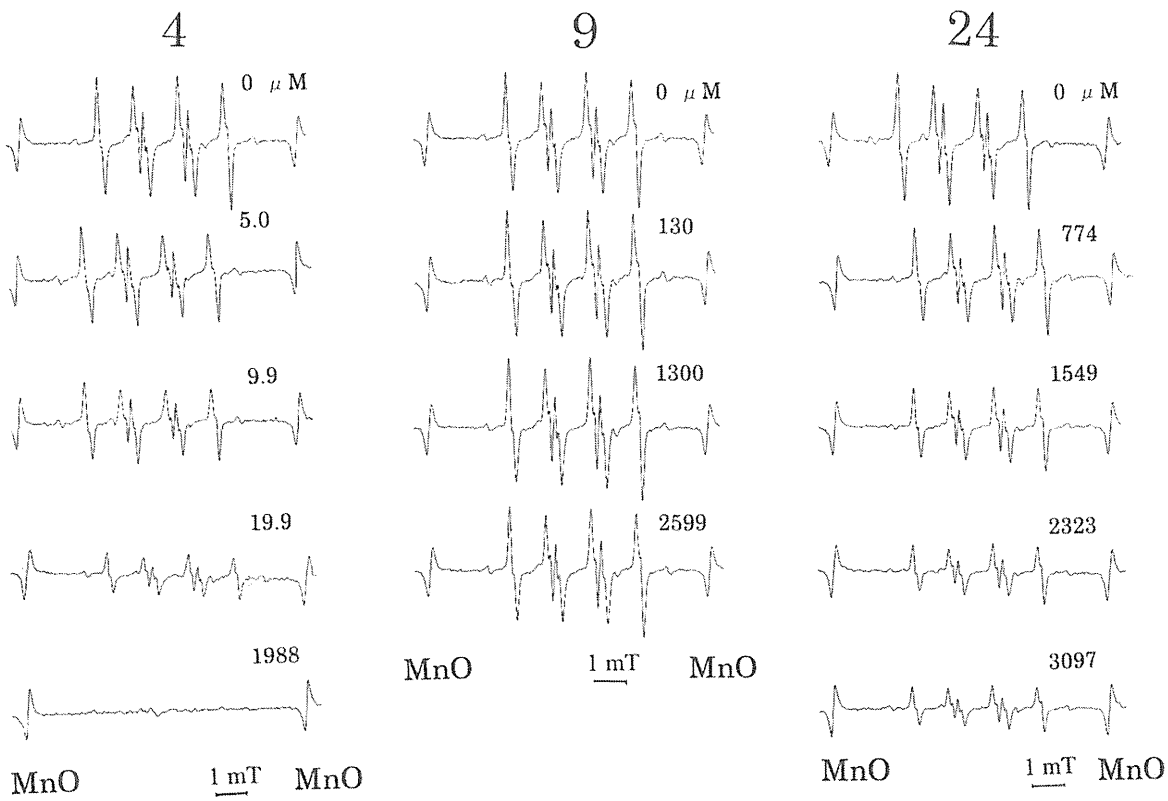

Figure 5. *ESR spectra of DMPO-OOH adduct produced by HX-XOD reaction mixture in the presence of the indicated concentrations of 2-acetylaminoazulene* ***[4]****, diethyl 2-chloroazulene-1, 3-dicarboxylate* ***[9]*** *or methyl 7-isopropyl-2-methoxyazulene-1-carboxylate* ***[24]****.*

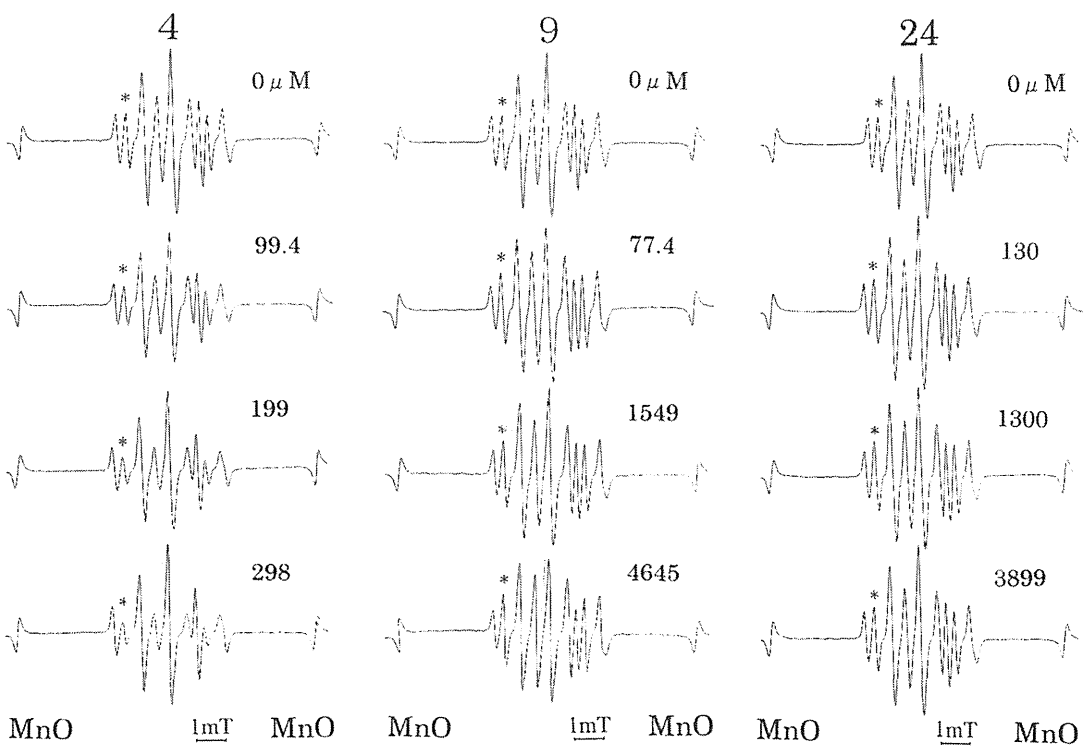

Figure 6. *ESR spectra of carboxy-PTIO + NOC-7 in the presence of the indicated concentrations of 2-acetylaminoazulene* ***[4]****, diethyl 2-chloroazulene-1,3-dicarboxylate* ***[9]*** *or methyl 7-isopropyl-2-methoxyazulene-1-carboxylate* ***[24]****. Measured at 3 minutes. The second peak (indicated by asterisks)(derived from carboxy-PTI) was used for the calculation of NO radical intensity.*

The present study demonstrated, for the first time, that methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** induced apoptotic cell death characterized by caspase 3 activation and DNA fragmentation. At present, the mechanism by which methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** activated caspase 3 is unclear, since this compound did not significantly activate caspase 8 (mitochondria-independent extrinsic pathway) and caspase 9 (mitochondria-dependent intrinsic pathway) (37). We found, by observation under light microscope (data not shown), that a very low percentage of the cells treated with this compound produced apoptotic bodies, possibly inducing necrotic cell populations.

Our ESR study demonstrated that 2-acetylaminoazulene **[4]** produced radical and scavenged O_2^- and NO more efficiently than diethyl 2-chloroazulene-1,3-dicarboxylate [9] and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]**, which did not produce radical and poorly scavenged these radical species. This suggests that the two different activities, that is radical production and scavenging, are present in the same molecule. The present study suggests that methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** may induce apoptotic cell death by a mechanism in which radical is not involved, whereas 2-acetylaminoazulene **[4]** may induce cell death by a radical-mediated oxidation mechanism. In a parallel study with 27 tropolone derivatives, we found that 5-aminotropolone, which produced a higher amount of radical and more efficiently scavenged O_2^- and NO than azulenes, induced apoptosis at a much lower concentration (38). Further studies are underway to elucidate the mechanism by which azulenes induce apoptosis in tumor cell lines.

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