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Tumor-specific Cytotoxicity and Type of Cell Death Induced by Benzo[*b*]cyclohept[*e*][1,4]oxazine and 2-Aminotropone Derivatives

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Abstract. *A total of twenty benzo[b]cyclohept[e][1,4]oxazines and their S-analogs, and 2-aminotropone derivatives were investigated for their cytotoxicity against three human normal cells and four tumor cell lines. These compounds showed moderate tumor-specific cytotoxicity. The cytotoxicity was enhanced by bromination at the tropone ring and replacement by formylbenzene. The cytotoxicity of 2-(2-hydroxyanilino) tropone was enhanced by introduction of bromine or isopropyl group to the tropone ring. The presence of a hydroxyl group at ortho or para-position should be necessary for the appearance of cytotoxicity and tumor-specificity. The highly active derivatives, 7-bromo-2-(4-hydroxyanilino)tropone* ***[16]*** *and 4-isopropyl-2-(2-hydroxyanilino)tropone* ***[20]****, induced internucleosomal DNA fragmentation and caspase-3, -8 and -9 activation in human promyelocytic leukemia HL-60 cells, but only at concentrations twice or four times higher than CC_{50} values. These compounds induced no discernible DNA fragmentation, and activated caspases much more weakly in human oral squamous cell carcinoma HSC-2 cells. Both* ***[16]*** *and* ***[20]*** *failed to induce the production of acidic organelles, a marker of autophagy, in contrast to the nutritional starvation. These data demonstrated that 2-aminotropones showed relatively higher tumor-specificity than benzo[b]cyclohept[e] [1,4]oxazine, and that 2-aminotropones induced little or no apoptotic cell death in oral squamous cell carcinoma, in contrast to HL-60 cells.*

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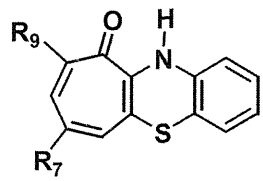
Heterocylic compounds are known to display diverse biological activities (1). Hinokitiol and its related derivatives with a tropolone skeleton (2-4) have been reported to exhibit various biological activities such as antimicrobial (5), antifungal (6) and phytogrowth-inhibitory activity (7, 8), cytotoxic effects on mammalian tumor cells (9, 10), and inhibitory effects on catechol-*O*-methyltransferase (11) and metalloproteases (5). Hinokitiol acetate did not exhibit cytotoxic activity (10), antimicrobial activity nor metalloprotease inhibition (5), suggesting that these biological effects of hinokitiol-related compounds may result from the formation of metal chelation by the carbonyl group at C-1 with the hydroxyl group at C-2 in the tropone skeleton. A total of 153 azulene, tropolone and azulenequinone-related compounds have been investigated for their ability to induce tumor-specific cytotoxicity and to inhibit NO and PGE_2 production by lipopolysaccharide (LPS)-stimulated mouse macrophage RAW264.7 cells (12-24).

Differing from unreactive heterocyclic-annulated tropylium compounds, benzo[*b*]cyclohept[*e*][1,4]oxazines and their *S*-analogs are generally very reactive, especially towards 1,4-difunctional nucleophiles such as *O*-phenylenediamine, ethylenediamine and their *S*- and *O*-analogues (25). It has recently been reported that among 26 benzocycloheptoxazines, 6,8,10-triboromobenzo [*b*]cyclohepta[*e*][1,4]oxazine, 6-bromo-2-methoxybenzo[*b*] cyclohepta[*e*][1,4]oxazine and 6-bromo-2-chlorobenzo[*b*] cyclohepta[*e*][1,4]oxazine showed the highest tumor specificity, and induced apoptosis in human promyelocytic leukemic HL-60 cells, but not in human oral squamous cell carcinoma HSC-4 cells, suggesting a diversity of type of cell death induced in human tumor cell lines (22). In order to further extend this study, a total of twenty benzo[*b*]cyclohept[*e*][1,4]oxazines and their *S*-analogs, and 2-aminotropone derivatives (Figure 1, Table I) were investigated for their tumor-specific cytotoxicity and apoptosis-inducing activity against three human normal cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast



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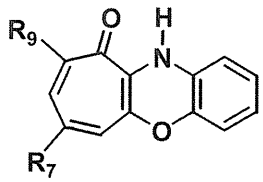

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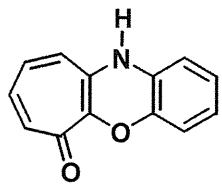

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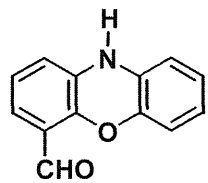

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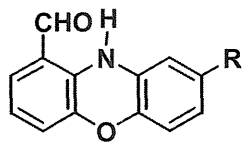

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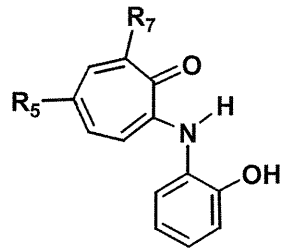

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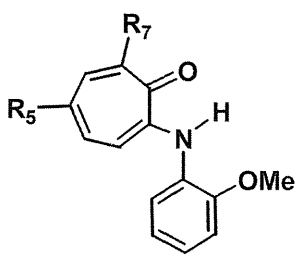
10: R_5=H; R_7=Br
11: R_5=Br; R_7=H
12: R_5, R_7=Br
18: R_5=*i*Pr; R_7=H



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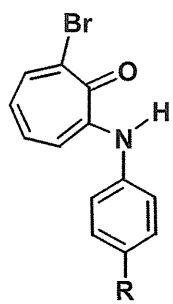
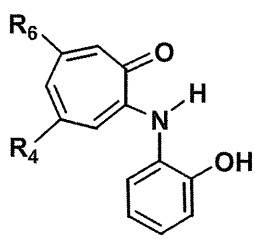
14: R_5=Br; R_7=H
15: R_5, R_7=Br



16: R=OH
17: R=Me



19: R_4=H; R_6=*i*Pr
20: R_4=*i*Pr; R_6=H

Figure 1. *Chemical structure of benzo[b]cyclohepta[e][1,4]benzoxazines and their S-analogs* ***[1-8]****, and 2-aminotropones* ***[9-20]****.*

HPLF) and four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4 and promyelocytic leukemia HL-60).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Gland Island, NY, USA); RPMI 1640 medium, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan).

Synthesis of benzo[b]cyclohept[e][1,4]oxazines and their S-analogues, and 2-aminotropolones. Benzo[*b*]cyclohept[*e*][1,4]oxazines and their *S*-analogs **[1-8]** and 2-aminotropone derivatives **[9-20]** (Figure 1, Table I) were synthesized, according to methods previously published (26-36).

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.

Table I. *Benzo[b]cyclohepta[e][1,4]benzoxazines and their S-analogues* ***[1-8]****, and 2-aminotropolones* ***[9-20]*** *synthesized in this study.*

Compound	Name	Method used (ref.)
[1]	benzo[*b*]cyclohepta[*e*][1,4]thiazin-10(11*H*)-one	25-28
[2]	7,9-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazin-10(11*H*)-one	25, 28
[3]	benzo[*b*]cyclohept[*e*][1,4]oxazin-10(11*H*)-one	25, 27-34
[4]	7,9-dibromobenzo[*b*]cyclohept[e][1,4]oxazin-10(11*H*)-one	25,32
[5]	benzo[*b*]cyclohept[*e*][1,4]oxazin-6(11*H*)-one	25, 31, 33
[6]	4-formylphenoxazine	25, 31, 33, 35
[7]	1-formylphenoxazine	25, 31, 33, 35
[8]	8-chloro-1-formylphenoxazine	25, 33
[9]	2-(2-hydroxyanilino)tropone	25, 32-34
[10]	7-bromo-2-(2-hydroxyanilino)tropone	25, 30-33
[11]	5-bromo-2-(2-hydroxyanilino)tropone	25, 32, 36
[12]	5,7-dibromo-2-(2-hydroxyanilino)tropone	32, 36
[13]	7-bromo-2-(2-methoxyanilino)tropone	30, 32
[14]	5-bromo-2-(2-methoxyanilino)tropone	32, 36
[15]	5,7-dibromo-2-(2-methoxyanilino)tropone	32, 36
[16]	7-bromo-2-(4-hydroxyanilino)tropone	32, 33
[17]	7-bromo-2-(4-methylanilino)tropone	32
[18]	5-isopropyl-2-(2-hydroxyanilino)tropone	25, 29
[19]	6-isopropyl-2-(2-hydroxyanilino)tropone	25, 29
[20]	4-isopropyl-2-(2-hydroxyanilino)tropone	25, 29

Human promyelocytic leukemic HL-60 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. Tumor cell lines were obtained from Riken Cell Bank. Normal cells were prepared from the periodontal tissues, according to the guideline of Institutional Board of Meikai University Ethic Committee (No. 0206), after obtaining the informed consent from the patients. Since HGF, HPC and HPLF cells have a limited lifespan due to *in vitro* senescence (37), these cells at the population doubling level of 5-8 were used for the present study.

Assay for cytotoxic activity. Cells (except for HL-60 cells) were inoculated at 3×10^3 cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 hours, the medium was removed by suction with aspirator, and replaced with 0.1 mL of fresh medium containing various concentrations of test compounds. The first well contained a 500 μM sample and was diluted 2-fold sequentially, with duplicate wells for each concentration. Cells were incubated for another 48 hours, and the relative viable cell number was then determined by 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 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) (20). A_{540} of control cells were usually in the range from 0.50 to 1.2.

HL-60 cells were inoculated at 5×10^4/0.1 mL in 96-microwell plates and various concentrations of test compounds were added. After incubation for 48 hours, the viable cell number was determined by trypan blue exclusion, under a light microscope.

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)]} × (4/3).

Assay for DNA fragmentation. HSC-2 cells (1.2×10^5) were inoculated onto 6-well plates (9.6 cm^2) and left for 48 hours to complete adherence to the plate. Adherent HSC-2 cells or HL-60 cells freshly prepared (5×10^5) were cultured for 6 or 24 hours in fresh culture medium (3 mL) without (control) or with ×1, ×2 or ×4 CC_{50} of 7-bromo-2-(4-hydroxyanilino)tropone **[16]** or 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]**. Cells were then harvested, washed once with PBS (-) and lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA-2Na, 0.5% (w/v) sodium *N*-lauryl-sarcosinate solution]. The lysate 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 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-2Na, pH 8.0). 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-2Na, pH 8.0). DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by UV irradiation (37) were run in parallel. After staining with ethidium bromide, DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc-It, UVP, Inc., Upland, CA, USA) (38).

Assay for caspase activation. HSC-2 cells (6×10^5) were inoculated on 8.5 cm-plates and incubated for 48 hours to allow for complete adherence. The adherent HSC-2 and HL-60 cells freshly prepared (3×10^6 in 6-well plate) were further incubated for 24 hours in fresh medium without (control) or with ×1, ×2 or ×4 CC_{50} **[16]** or **[20]**. Cells were washed with PBS(-) and lysed in lysis solution [50 mM Tris-HCl (pH 7.5), 0.3% NP-40, 1 mM DTT]. After standing for 10 minutes on ice and centrifugation for 5 minutes at 21,000 × g,

Table II. *Cytotoxicity of benzocycloheptoxazines and aminotropones on human tumor and normal cells.*

		CC_{50} (μM)							
		Human tumor cell lines				Human normal cells			
Compd.	MW	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
[1]	227	83	67	78	30	99	96	>100	1.5
[2]	385	21	38	18	17	53	68	98	3.1
[3]	211	110	107	117	73	116	118	>125	1.2
[4]	369	11	12	12	11	11	12	>13	1.0
[5]	211	163	152	206	73	341	423	408	2.6
[6]	211	74	47	67	22	93	109	115	2.0
[7]	211	38	33	31	24	43	47	50	1.5
[8]	246	>100	40	13	31	78	>100	>100	2.0
[9]	213	202	271	264	123	369	380	406	1.8
[10]	292	75	78	85	27	94	92	97	1.4
[11]	292	53	63	65	22	188	196	199	3.8
[12]	371	24	38	53	18	96	96	102	3.0
[13]	306	60	113	82	39	192	180	185	2.5
[14]	306	83	97	85	32	101	116	121	1.5
[15]	385	20	16	22	11	18	20	22	1.2
[16]	292	87	118	88	32	287	316	370	4.0
[17]	290	116	105	118	42	177	183	204	2.0
[18]	255	46	65	32	34	103	104	114	2.4
[19]	255	30	76	41	24	142	158	175	3.7
[20]	255	37	66	52	21	196	203	178	4.4

Each value represents mean from three independent experiments. S.D. <20%.

the supernatant was collected. Lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL lysis solution containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for 18 hours at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by a microplate reader (20).

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were imaged by staining of the cells with 0.1 μg/mL acridine orange for 20 minutes. Samples were then examined under Laser Scanning Microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany) using the following filters: excitation filter 488 nm, emission filter 505-530 nm and >650 nm (20).

Results

Cytotoxic activity of benzo[b]cyclohepta[e][1,4]thiazinones [1, 2] and oxazines [3-8]. First, cytotoxic activity of two benzo[*b*]cyclohepta[*e*][1,4]thiazinone **[1, 2]** (Figure 1) was investigated. The bromination at C-2- and C-4 of tropone ring significantly increased both cytotoxicity and tumor-specificity (TS=3.1 **[2]** *vs.* 1.5 **[1]**) (Table II). Following this, the cytotoxicity of three oxazines **[3-5]** (Figure 1) was investigated. Similarly, bromination at C-2- and C-4 **[4]** also increased the cytotoxicity of **[3]**, but without increase in the TS value (TS=1.2 *vs.* 1.0). However, the shift in the position of carbonyl group **[5]** from C-1 to C-4 slightly enhanced the tumor-specificity (TS=2.6). Three phenoxazines attached with a formyl group at different positions **[6-8]** (Figure 1) showed comparable cytotoxicity and tumor-specificity (TS=2.0, 1.5 and 2.0, respectively) (Table II). Among four tumor cell lines, HL-60 cells were the most sensitive (Table II).

Cytotoxic activity of 2-aminotropone related compounds [9-20]. Cytotoxic activity of 2-(2-hydroxyanilino)tropone [9] (Figure 1) was enhanced by bromination at C-7 **[10]**, C-5 **[11]**, or both at C-7 and C-5 **[12]** (Table II). The tumor-specific cytotoxicity of **[9]** (TS=1.8) was enhanced by introduction of one or two bromines **[11, 12]** (TS=3.8 and 3.0, respectively). The hydroxyl group seems to be important for tumor-specificity, since the replacement of the hydroxyl group by a methoxy group (**[14, 15]**) significantly reduced the tumor-specificity (TS=1.5 and 1.2, respectively). Although compound **[10]** showed minor tumor-specificity (TS=1.4), the shift of hydroxyl group from C-2 to C-4 (**[16]**) enhanced the tumor-specificity (TS=4.0). A hydroxyl group at C-4 should be essential for the higher tumor-specificity, since the tumor-specificity was apparently reduced by replacing this hydroxyl group with a methyl group **[17]** (TS=2.0). Addition of an isopropyl group to C-4, C-5 or C-6 of the tropone ring of compound **[9]** strongly enhanced the tumor-specificity

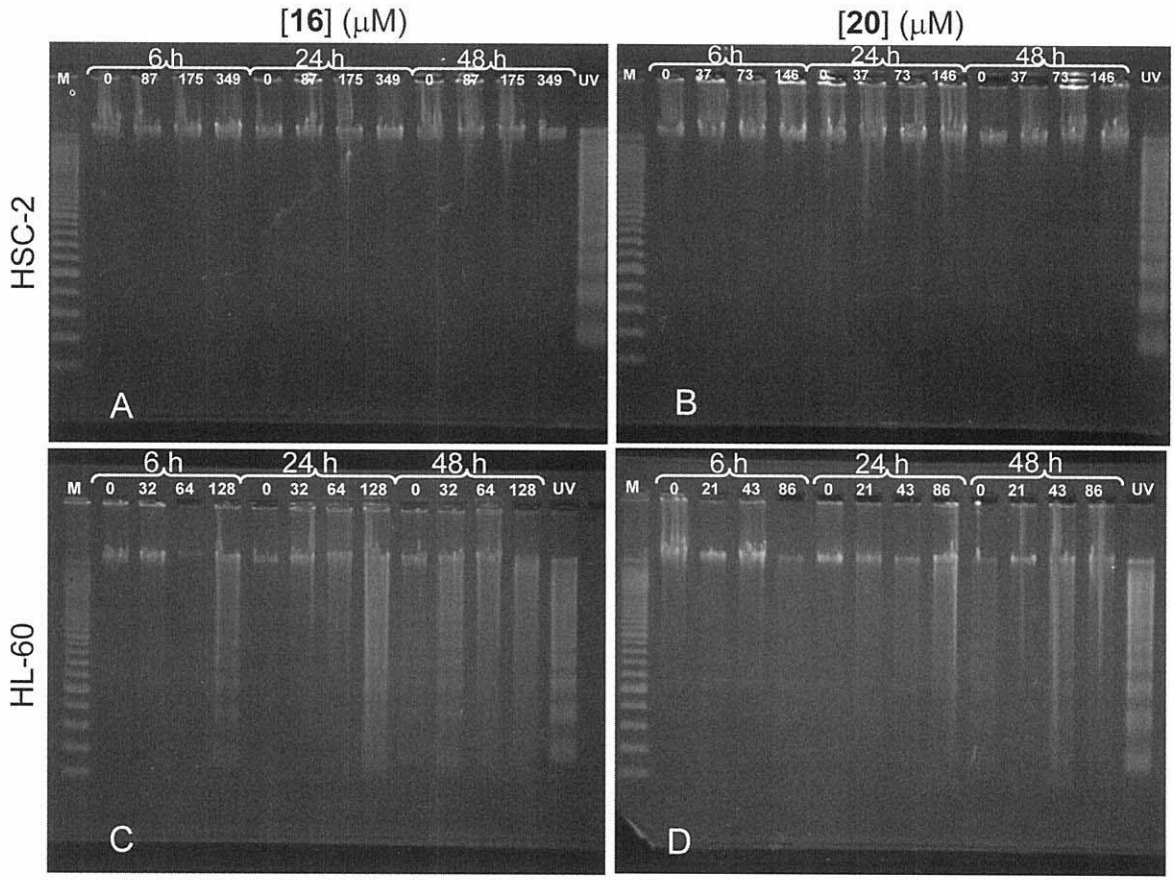


Figure 2. *Induction of DNA fragmentation by two 2-aminotropolones derivatives. Near confluent HSC-2 (A, B) and HL-60 cells (C, D) were incubated for 6, 24 or 48 hours with the indicated concentrations of* ***[16]*** *(A, C) or* ***[20]*** *(B, D). DNA was then extracted and applied to agarose gel electrophoresis. M: Marker DNA. UV, DNA from apoptotic HL-60 cells induced by UV irradiation (38). One representative data from three independent experiments are shown.*

(TS=2.4, 3.7 and 4.4, respectively) (Table II). Among four tumor cell lines, HL-60 cells were again the most sensitive (Table II).

Induction of apoptosis. Since 7-bromo-2-(4-hydroxyanilino) tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** showed the highest tumor-specificity among the twenty derivatives used in this study, these compounds were tested for apoptosis-inducing activity. Both of these compounds did not induce any traces of internucleosomal DNA fragmentation in HSC-2 cells at any concentrations or incubation times (Figure 2A, B). On the other hand, higher concentrations of these compounds (4 times that of CC_{50} values) induced internucleosomal DNA fragmentation in HL-60 cells at 6 and 24 hours after the treatment. When these cells were incubated for 48 hours, DNA fragmentation could be observed at their CC_{50} values (Figure 2C, D).

Further, 7-bromo-2-(4-hydroxyanilino)tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** did not induce the activation of caspase-3, caspase-8 and caspase-9 at CC_{50} values in HSC-2 cells (Figure 3A, B). These compounds induced caspase activation at twice or four times higher than the CC_{50} values, but to a much lesser extent than that observed in HL-60 cells (Figure 3C, D). Both **[16]** and **[20]** failed to induce the production of acidic organelles (Figure 4A-E), in contrast to the nutritional starvation in Hank's balanced salt solution (Figure 4F).

Discussion

The present study demonstrates that all of twenty benzo[*b*]cyclohepta[*e*][1,4]thiazines and their *S*-analogues, and 2-aminotropolone derivatives showed moderate tumor-specific cytotoxicity (TS=1.4-4.4). Their cytotoxic activity was enhanced by bromination at the tropone ring in all types of compounds: **[1]** *vs.* **[2]** in thiazines, **[3]** *vs.* **[4]** in oxazines, and **[9]** *vs.* **[10-17]** in 2-aminotropones. This is in consistency with previous reports that the substitution of

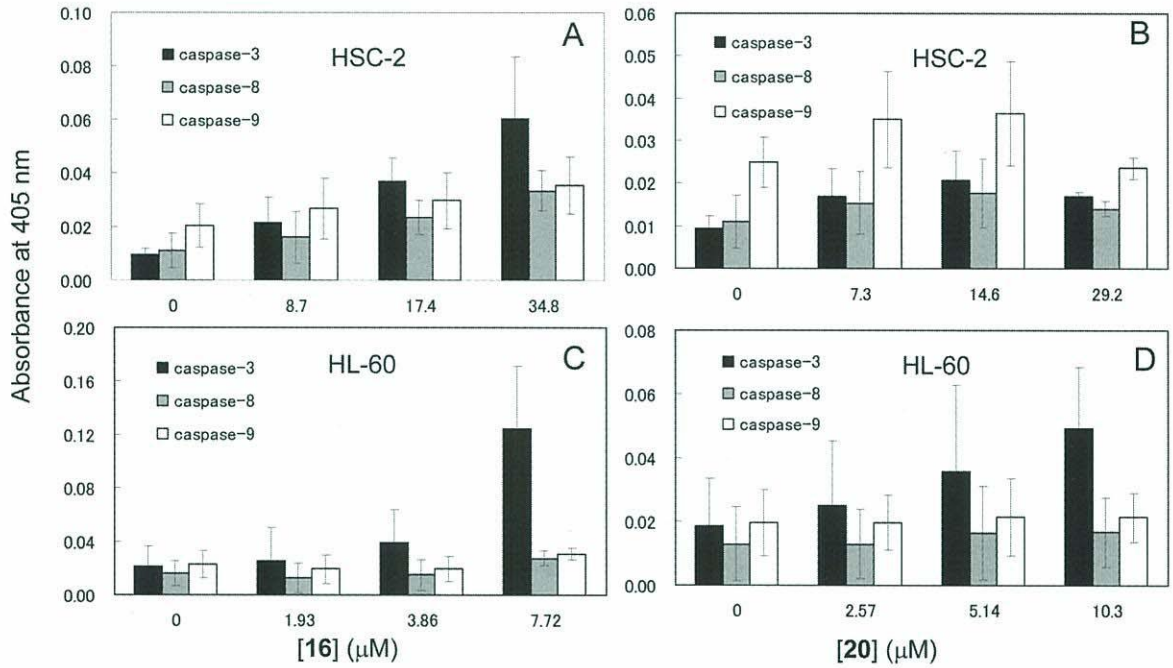


Figure 3. *Activation of caspases-3, -8 and -9 by two 2-aminotropolone derivatives. Near confluent HSC-2 (A, B) and HL-60 cells (C, D) were incubated for 24 hours with the indicated concentrations of* ***[16]*** *(A, C) or* ***[20]*** *(B, D). Each point represents mean±S.D. from 3~4 independent experiments.*

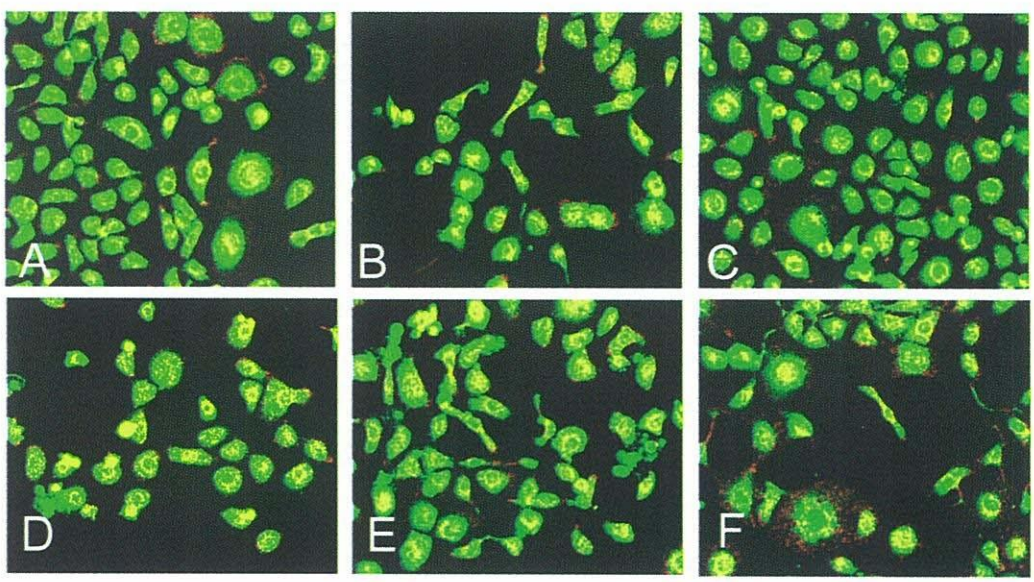


Figure 4. *Effect of compounds* ***[16]*** *and* ***[20]*** *on the formation of acid organelles. HSC-2 cells were incubated for 4 hours without (A) or with 87 (B) or 174 (C) μM [16], or 36.5 (D) or 73 (E) μM* ***[20]****. To induce autophagy, aliquots of the cells were cultured under starved condition in Hank's balanced salt solution (F). The cells were then stained with acridine orange and the formation of acid organelle was observed under confocal laser scanning microscope.*

chloride, bromide or iodine at the C-3 position of trifluoroacetylazulenes and trichloroacetylazulenes further enhanced their cytotoxic activity against four tumor cell lines, especially HL-60 cells (20).

Among twenty compounds tested in this study, 7-bromo-2-(4-hydroxyanilino)tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** showed the highest tumor-specific cytotoxicity. HL-60 cells were found to be

the most sensitive to these compounds. Both **[16]** and **[20]** induced apoptosis (characterized by internucleosomal DNA fragmentation and caspase activation) in HL-60 cells, but only at concentrations higher than CC_{50}. On the other hand, these compounds failed to induce internucleosomal DNA fragmentation in HSC-2 cells, only marginally activating the caspases. The non-apoptosis-inducing activity of these compounds might be a result of their structural characteristics, considering that they have an α,β-unsaturated ketone in the tropone ring (38). Similar observations that trihaloacetylazulenes induced the apoptotic cell death in HL-60 cells as well as autophagic cell death (characterized by lower activation of caspases, lack of DNA fragmentation, vacuolization and autophagosome formation) in HSC-4 cells have also been reported (39). This suggests a connection between the induction of non-apoptosis and drug resistance, and also the potential applicability of 2-aminotropolones for the treatment of leukemia.

In conclusion, the present study demonstrated that 2-aminotropones showed higher tumor-specificity than benzo[*b*]cyclohept[*e*][1,4]oxazine, and that 2-aminotropones induced little or no apoptotic cell death (characterized by weak caspase activation and no internucleosomal DNA fragmentation) without induction of secondary lysosome (marker for autophagy). Further experiments are required to clarify the type of cell death induced in oral squamous cell carcinoma cell lines.

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