Tumor-specific Cytotoxicity and Type of Cell Death Induced by Benzocycloheptoxazines in Human Tumor Cell Lines

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Abstract. *Twenty-six benzocycloheptoxazine derivatives 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 (squamous cell carcinoma HSC-2, HSC-3, HSC-4, promyelocytic leukemia HL-60). Benzo[b]cyclohepta[e][1,4]thiazine* ***[1]*** *exhibited very weak cytotoxicity, whereas its 6,8,10-tribromo derivative* ***[3]*** *exhibited higher cytotoxicity and tumor specificity (TS=5.6). 6H-Benzo[b]cyclohepta[e][1,4]diazine* ***[4]*** *and its cation* ***[5]*** *exhibited no tumor specificity. Among eighteen benzo[b]cyclohepta[e][1,4]oxazine derivatives* ***[6-23]****, 6,8,10-triboromo-* ***[9]****, 6-bromo-2-methyl-* ***[20]****, and 6-bromo-2-chloro-* ***[21]*** *derivatives showed the highest tumor-specific cytotoxicity (TS=12.5, 9.1 and 11.5, respectively). 14H-[1,4]Benzoxazino[3',2':3,4]cyclohepta[1,2-b][1,4]benzoxazine* ***[24]*** *and its 7-bromo-* ***[25]*** *and 7-isopropyl-* ***[26]*** *derivatives had much lower cytotoxicity and tumor-specificity. Compounds* ***[9, 20, 21]*** *at 50% cytotoxic concentration (CC_{50}) induced internucleosomal DNA fragmentation and caspase activation in HL-60 cells. On the other hand, these compounds induced apoptosis only at concentrations higher than CC_{50} in HSC-2 cells and failed to induce apoptosis in HSC-4 cells. Compounds* ***[9, 20, 21]*** *induced the formation of acidic organelles as measured by acridine orange staining. Transmission electron microscopy demonstrated the induction of moderate enlargement of mitochondria, the endoplasmic reticulum and nuclear membrane, and the vacuolation of the endoplasmic reticulum and the presence of a number of lamellar body-like organelles. These results indicate the diversity of the type of cell death induced by benzocycloheptoxazine derivatives in human tumor cell lines.*

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Hinokitiol and related compounds with a tropolone skeleton (1-3) have been reported to exhibit various biological activities such as antimicrobial (4), antifungal (5), and phytogrowth-inhibitory activities (6, 7), cytotoxic effects on mammalian tumor cells (8, 9), and inhibitory effects on catechol-*O*-methyltransferase (10) and metalloproteases (4). Hinokitiol acetate did not exhibit cytotoxic activity (9), antimicrobial activity or metalloprotease inhibition (4), suggesting that these biological effects of hinokitiol-related compounds may result from the metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 in the tropolone skeleton. Tropolone derivatives with a phenolic OH group, hinokithiol, its tosylate and methyl ethers have exhibited higher tumor-specific cytotoxic activity, while 2-aminotropolone showed the highest tumor-specificity and induced apoptosis in the human promyelocytic leukemic HL-60 cell line, possibly by radical-mediated redox reaction (11). 2,4-Dibromo-7-methoxytropone inhibited nitric oxide production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells *via* the inhibition of inducible nitric oxide synthase (iNOS) expression, rather than *via* the radical-mediated mechanism (12).

Differing from common, unreactive heterocyclic-annulated tropylium compounds, cyclohepta[*b*][1,4]benzoxazines and their *S*- and *O*-analogues are usually very reactive, especially towards 1,4-difunctional nucleophiles such as *O*-phenylenediamine, ethylenediamine and their *S*- and *O*-analogues (13). We therefore investigated here 26 benzocycloheptoxazines derivatives (Figure 1) for their tumor-

Table I. *Benzocycloheptoxazine derivatives synthesized in this study.*

Compound number	Name	Method used (Reference)
[1]	benzo[*b*]cyclohepta[*e*][1,4]thiazine	14-18
[2]	6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine	14, 15, 17
[3]	6,8,10-tribromobenzo[*b*]cyclohepta[*e*][1,4]thiazine	14, 15, 17
[4]	6*H*-benzo[*b*]cyclohepta[*e*][1,4]diazine	17-19
[5]	6*H*-benzo[*b*]cyclohepta[*e*][1,4]diazine cation	17-19
[6]	benzo[*b*]cyclohepta[*e*][1,4]oxazine	20-22
[7]	6-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	23-27
[8]	6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	26, 28-30
[9]	6,8,10-tribromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	26, 28-30
[10]	8-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	26, 28-30
[11]	7-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 30
[12]	9-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 30
[13]	8-isopropylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	21, 22
[14]	9-isopropylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	21, 22
[15]	6-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	27, 30
[16]	8-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	30
[17]	8-bromo-6-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	30
[18]	9-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	30
[19]	2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 25
[20]	6-bromo-2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 25
[21]	6-bromo-2-chlorobenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 25
[22]	8-bromo-2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 28
[23]	8-bromo-2-chlorobenzo[*b*]cyclohepta[*e*][1,4]oxazine	22, 28
[24]	14*H*-[1,4]benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine	22-25, 27
[25]	14*H*-7-bromo[1,4]benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine	28, 29
[26]	14*H*-7-isopropyl[1,4]benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine	22, 29

specific cytotoxicity and apoptosis-inducing activity against three normal human 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).

Materials and Methods

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

Synthesis of benzocycloheptoxazines. Benzocycloheptoxazine derivatives were synthesized according to methods previously published (Table I).

Cell culture. Three human oral tumor cell lines (HSC-2, HSC-3, HSC-4) and three normal human 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 Riken Cell Bank Japan. Normal cells were prepared from periodontal tissues, according to the guideline of the Institutional Board of Meikai University Ethics Committee (No. 0707) after obtaining informed consent from the patients. Since HGF, HPC and HPLF cells have a limited lifespan due to *in vitro* senescence (31), these cells were used for the present study at a population doubling level of 5-8.

Assay for cytotoxic activity. Cells (other than HL-60 cells) were inoculated at $5x10^3$ cells/well in 96-microwell plates (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 different concentrations of the test compounds. Each test compound was dissolved in DMSO at a concentration of 50 mM. The first well contained 500 μM sample and was then diluted 2-fold sequentially, with 3 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(–)) which was replaced with fresh culture medium containing 0.2 mg/mL MTT and cells were incubated for another 4 hours. The cells were lysed with 0.1 mL of DMSO and the absorbance of the cell lysate at 540 nm A_{540} was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland) (32). The A_{540} of control cells was usually in the range from 0.40 to 0.90.

Table II. *Cytotoxic activity of benzocycloheptoxazines against human normal and tumor cells.*

		Cytotoxic activity (CC_{50}: M)							
		Normal human cells			Human tumor cell lines				
Compound	MW	HGF	HPC	HPLF	HSC-2	HSC-3	HSC-4	HL-60	TS
[1]	211.1	>500.0	>500.0	499.6	486.1	449.2	>500.0	149.1	1.3
[2]	369	23.2	77.2	24.4	87.9	51.5	60.2	9.6	0.8
[3]	448	63.9	87.2	95.2	11.1	13.5	18.5	15.1	5.6
[4]	194	73.6	84.2	177.6	25.3	36.8	46.7	13.7	3.7
[5]	254	>500.0	>500.0	>500.0	>500.0	449.3	>500.0	406.2	1.1
[6]	195	349.0	369.7	379.8	264.5	202.3	328.8	42.7	1.7
[7]	273.9	74.8	97.4	106.9	37.2	16.3	75.6	12.7	2.6
[8]	352.8	451.3	397.2	414.0	267.4	165.6	319.7	231.4	1.7
[9]	431.7	406.9	383.6	>500.0	28.6	47.0	34.8	27.4	12.5
[10]	273.9	278.1	310.8	176.0	90.0	90.0	142.2	34.5	2.9
[11]	273.9	126.7	128.9	190.4	83.3	57.2	123.2	10.7	2.2
[12]	273.9	71.2	69.2	156.1	71.8	53.7	91.9	36.9	1.6
[13]	236	298.1	263.1	310.9	51.3	38.3	79.4	18.0	6.2
[14]	236	173.7	273.1	232.4	109.2	70.9	158.5	27.8	2.5
[15]	253	301.1	360.0	376.7	84.0	63.1	174.1	22.4	4
[16]	253	11.3	41.1	39.3	18.9	15.2	25.6	6.6	1.8
[17]	331.9	356.3	144.4	179.9	57.1	43.4	84.5	11.0	4.6
[18]	253	>500.0	>500.0	>500.0	209.6	140.8	352.5	58.3	2.6
[19]	209	157.1	230.1	186.1	91.9	65.9	134.7	19.8	2.4
[20]	287.9	167.7	103.1	149.9	16.4	13.3	26.0	6.1	9.1
[21]	308.4	124.9	358.6	449.6	25.2	21.5	47.9	13.8	11.5
[22]	287.9	136.4	338.8	284.4	100.0	62.0	179.8	141.0	2.1
[23]	308.4	346.7	366.6	418.6	72.1	60.4	236.8	20.8	3.9
[24]	300	>500.0	>500.0	>500.0	>500.0	442.8	>500.0	326.4	1.1
[25]	378.9	>500.0	>500.0	>500.0	444.5	344.0	430.5	67.6	1.6
[26]	342	>500.0	>500.0	>500.0	>500.0	450.3	>500.0	148.2	1.3

Each value represents the mean from three independent experiments.

HL-60 cells were inoculated at $7.5x10^4$ cells/0.1 mL in 96-microwell plates and different 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:

$$TS=\{[CC_{50}\,(HGF) + CC_{50}\,(HPC) + CC_{50}\,(HPLF)] / [CC_{50}\,(HSC\text{-}2) + CC_{50}\,(HSC\text{-}3) + CC_{50}\,(HSC\text{-}4) + CC_{50}\,(HL\text{-}60)]\} \times (4/3).$$

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 subjected to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by 1 μg/mL actinomycin D (Act D) were run in parallel (32). After staining with ethidium bromide, DNA was visualized by UV irradiation and photographed by a CCD camera (Bio Doc-Ic; UVP, Inc., Upland, CA, USA).

Assay for caspase activation. Cells were washed with PBS(–) and lysed in lysis buffer [50 mM Tris-HCl (pH7.5) 0.3% NP-40, 1 mM DTT]. After standing for 10 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 of the lysis buffer containing substrates for caspase-3 (DEVD-*p*-nitroanilide (*p*NA)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA) (Kamiya Biochem Co., Seattle, WA, USA). After incubation for 2 hours at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by a plate reader (32).

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co., St. Louis, MO, USA) as described elsewhere (32). HSC-4 cells were stained with 1 μg/mL acridine

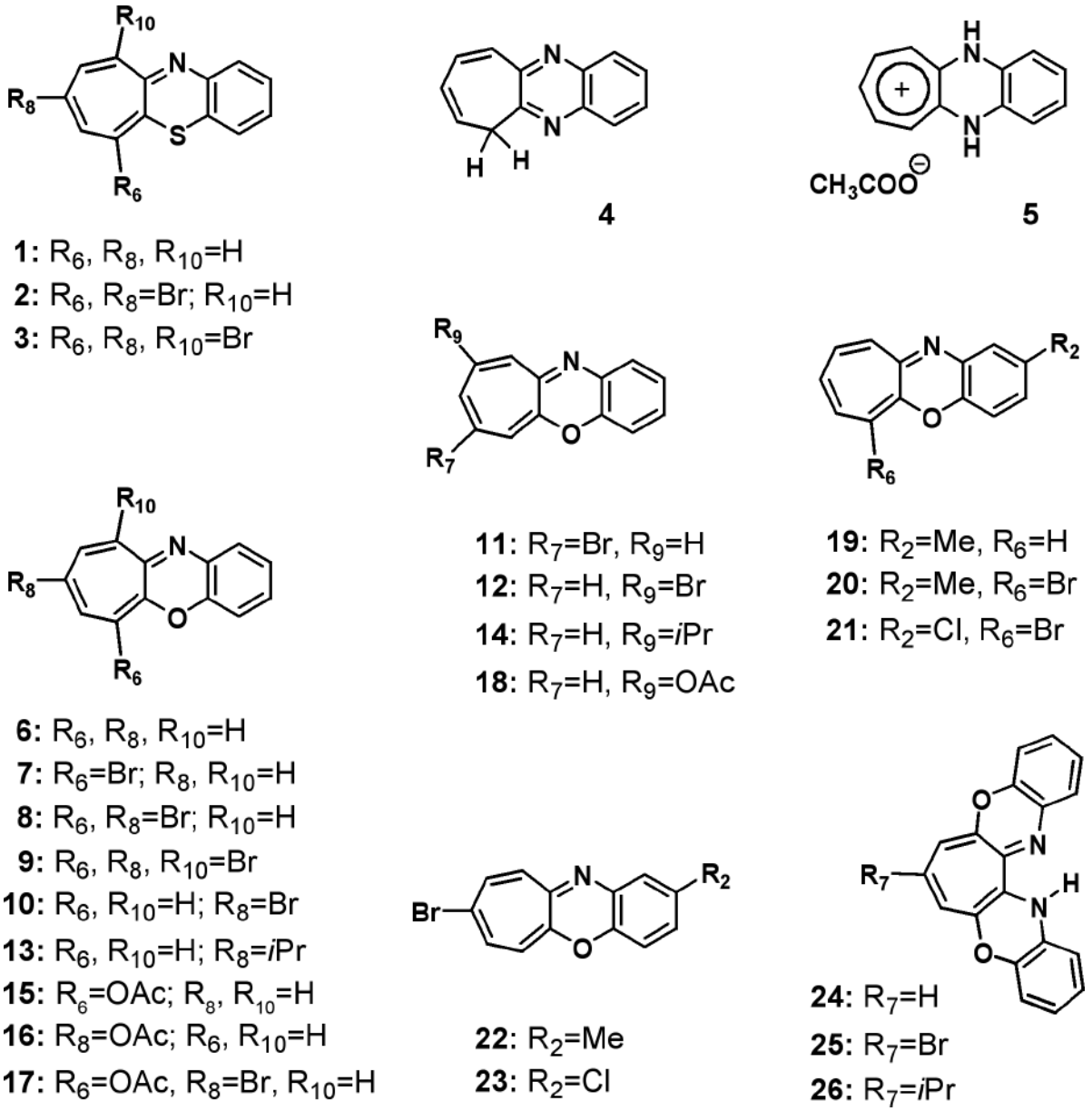

Figure 1. *Structures of the 26 benzocycloheptoxazines studied.*

orange for 15 minutes. Samples were then examined under a Laser Scanning Microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany), using the following filters: excitation 488 nm, emission 505-530 nm and >650 nm.

Electron microscopy. Cells were harvested by 0.25% trypsin-0.025%EDTA in PBS(–), and pelleted by centrifugation at 1,000 rpm for 5 minutes. The cells were fixed for 1 hour with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, postfixed for 1 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, Switzerland). 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 (32).

Results

Structure and activity relationship. Benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]** showed very weak cytotoxic activity against both normal and tumor cells (CC_{50}>500 μM), yielding essentially no tumor-specific cytotoxicity (TS=1.3) (Table II). The introduction of two bromines at the C-6 and -8

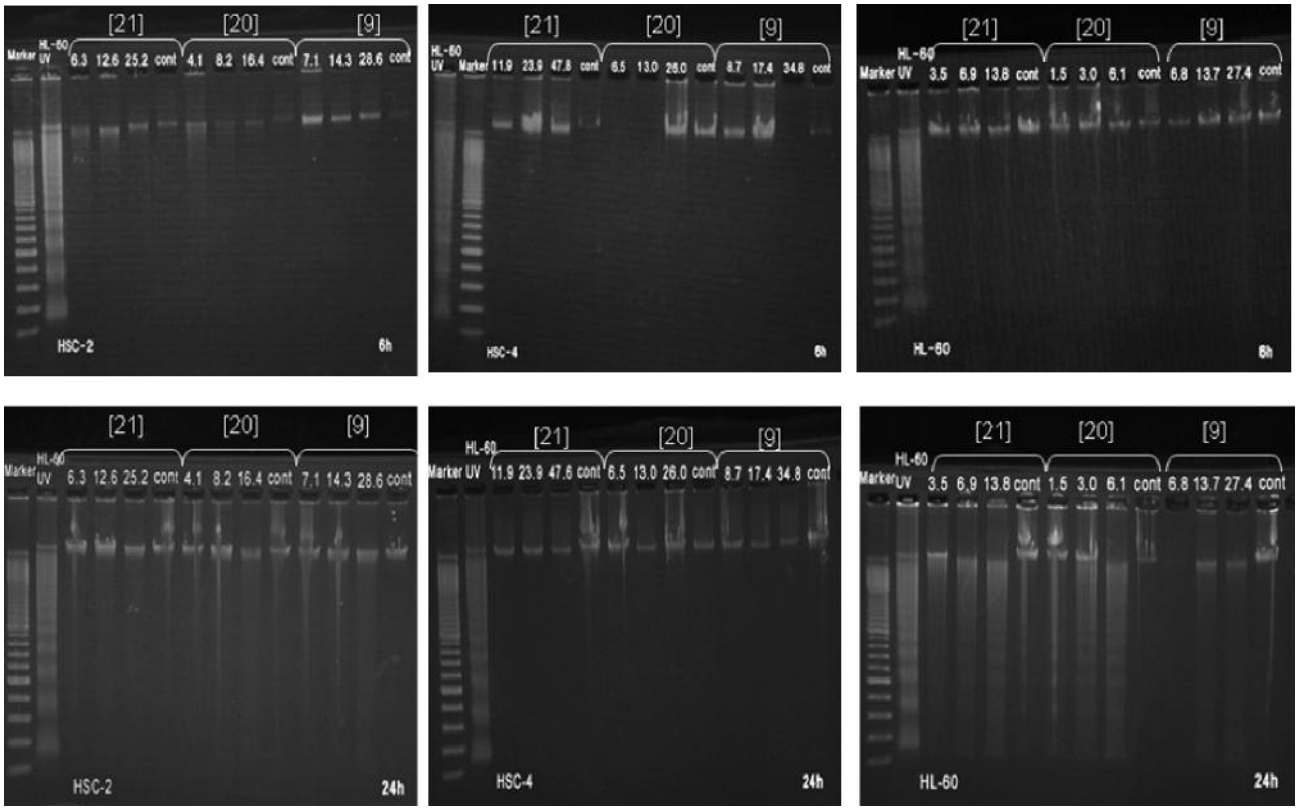

Figure 2. *Effect of three benzocycloheptoxazine derivatives **[9, 20, 21]** on the induction of DNA fragmentation in three human tumor cell lines. HSC-2 (left), HSC-4 (center) and HL-60 cells (right) were incubated for 6 (upper panel) or 24 hours (lower panel) with the indicated concentrations (μM) of **[9]**, **[20]** or **[21]**. DNA was then extracted and submitted to agarose gel electrophoresis. Marker DNA and DNA from apoptotic HL-60 cells induced by UV irradiation were also run. Representative data from one of three independent experiments are shown.*

positions of the cycloheptane ring **[2]** considerably enhanced cytotoxicity against both normal and tumor cells, and therefore did not increase the tumor-specificity (TS=0.8). The introduction of three bromines at the C-6, -8, and -10 positions of the cycloheptane ring **[3]**, enhanced the cytotoxicity against tumor cells to a greater extent, enhancing the tumor-specificity (TS=5.6).

6*H*-Benzo[*b*]cyclohepta[*e*][1,4]diazine **[4]** had higher cytotoxicity than benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]** and expressed some tumor-specificity (TS=3.7). The 6*H*-bnzo[*b*]cyclohepta[*e*][1,4]diazine cation **[5]** essentially had no cytotoxicity against normal or tumor cells (CC_{50}>500 μM) and had low tumor-specificity (TS=1.1).

Benzo[*b*]cyclohepta[*e*][1,4]oxazine **[6]** showed weak cytoxicity and tumor-specificity (TS=1.7). The introduction of one bromine at C-6 of the cycloheptane ring **[7]** significantly enhanced the cytotoxicity, but but not particularly increase the tumor-specificity (TS=2.6). The introduction of two bromines at C-6 and -8 of the cycloheptane ring **[8]** did not increase either cytotoxicity or tumor-specificity (TS=1.7). It should be noted that the introduction of three bromines at C-6, -8, and -10 of the cycloheptane ring **[9]** selectively enhanced the cytotoxicity against tumor cells, significantly enhancing the tumor-specificity (TS=12.5). However, the introduction of only one bromine at C-7 **[11]**, -8 **[10]** or -9 of the cycloheptane ring **[12]** slightly enhanced the cytotoxicity, but did not particularly increase the tumor-specificity (TS=2.2, 2.9 and 1.6, respectively). The introduction of an isopropyl group at C-8 **[13]** or -9 of the cycloheptane ring **[14]** slightly enhanced the tumor-specificity (TS=6.2 and 2.5, respectively). The introduction of an acetoxy group at C-6 **[15]**, -8 **[16]** or -9 **[18]**, or that of bromine at C-8 and an acetoxy group at C-6 **[17]** slightly enhanced the tumor-specificity (TS=4.0, 1.8, 2.6 and 4.6, respectively). The introduction of a methyl group at C-2 of the benzene ring [19] marginally enhanced the tumor-specificity (TS=2.4). It should be noted that the introduction of bromine at C-6 and a methyl group at C-2 **[20]**, or that of bromine at C-6 and chlorine at C-2 **[21]** greatly enhanced the tumor-specificity (TS=9.1 and 11.5, respectively). On the other hand, the introduction of bromine at C-8 and a methyl group at C-2

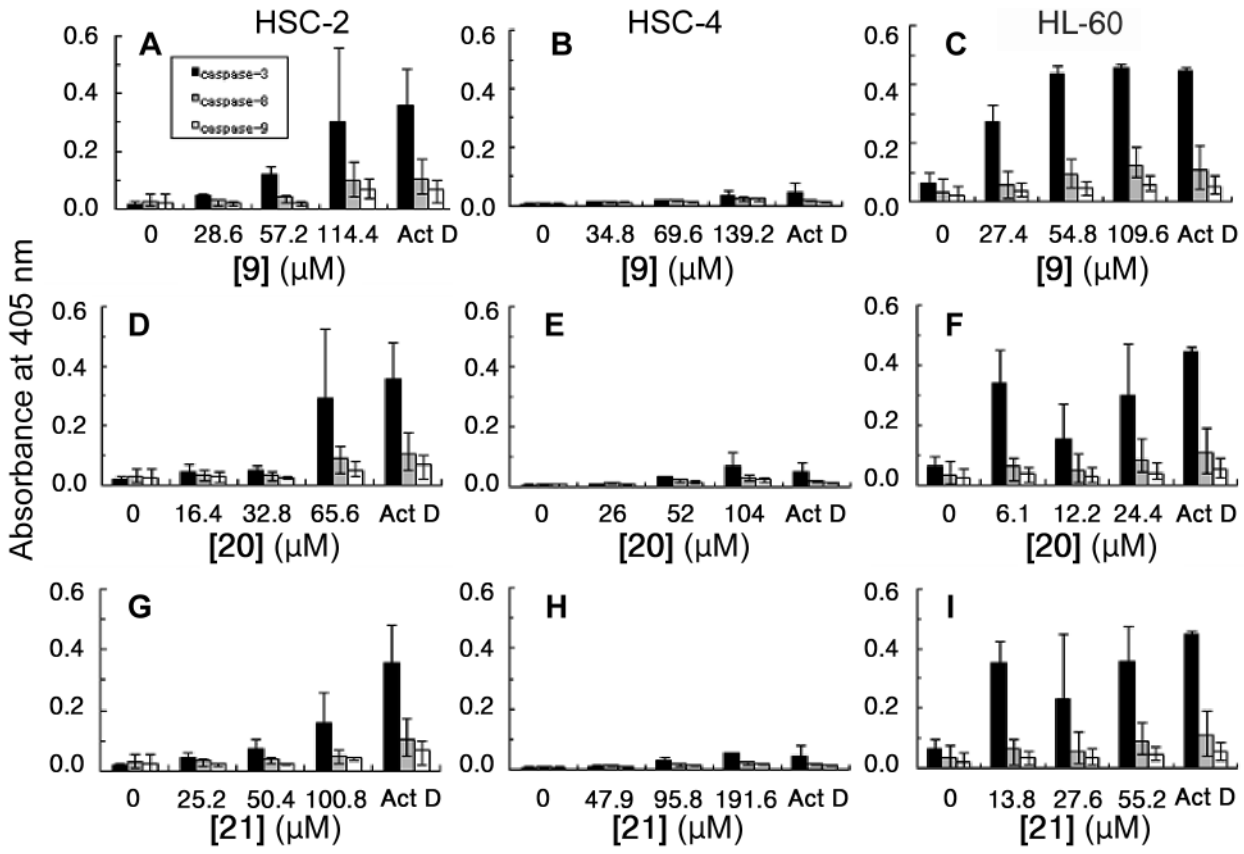

Figure 3. *Effect of three benzocycloheptoxazine derivatives **[9, 20, 21]** on caspase activation in three human tumor cell lines. HSC-2 (left), HSC-4 (center) and HL-60 cells (right) were incubated for 6 hours with the indicated concentrations of **[9]**, **[20]** or **[21]** or 1 μg/mL actinomycin D (Act-D) (positive control). Each point represents the mean±S.D. from 3-4 independent experiments.*

[22], and that of bromine at C-8 and chlorine at C-2 **[23]** only slightly enhanced the tumor-specificity (TS=2.1 and 3.9, respectively).

14*H*-[1,4]Benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4] benzoxazine **[24]** and its 7-bromo- **[25]** and 7-isopropyl- **[26]** derivatives had much lower cytotoxicity and tumor-specificity (TS=1.1, 1.6 and 1.3, respectively) (Table I).

Drug sensitivity of cell lines. Among the four tumor cell lines, HL-60 cells were generally the most sensitive, followed by HSC-2 cells. HSC-3 and HSC-4 cells were the most resistant.

Type of cell death induced. We next investigated the type of cell death induced by the most tumor-selective compounds **[9, 20, 21]**. When HL-60 cells were incubated with any of these compounds near their CC_{50}, internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, was not observed at 6 hours after treatment, but it became detectable at 24 hours after the treatment (Figure 2). These compounds also induced DNA fragmentation in HSC-2 cells, at the concentration of CC_{50}, whereas they failed to induce DNA fragmentation in HSC-4 cells (Figure 2).

Compounds **[9, 20, 21]** when used near the CC_{50} also activated caspase-3, caspase-8 and caspase-9 in HL-60 cells. They also activated these caspases in HSC-2 cells, but only at concentrations higher than the CC_{50}, whereas they failed to activate caspases in HSC-4 cells (Figure 3).

Compounds **[9, 20, 21]** induced the formation of acidic organelles easily recognizable as red dot-like structures after staining with acridine orange (Figure 4). To confirm the occurrence of autophagy, we investigated the changes of the fine cell structure on treatment with these compounds by transmission electron microscopy (Figure 5). When HSC-2 cells were cultured by compound **[9]** at the CC_{50} (29 μM), moderate enlargement of mitochondria and the endoplasmic reticulum was observed (Figure 5B). When HSC-2 cells were cultured with compound **[9]** at a concentration twice or quadruplicate that of the CC_{50} (57 or 114 μM), the enlargement of the endoplasmic reticulum was more evident (Figure 5C, D) than that observed at CC_{50} (29 μM), providing the vacuole-like profiles. The nuclear membrane was also enlarged in places (Figure 5C, D). Findings similar to these found with compound **[9]** were observed in the cells cultured with compound **[20]** (Figure 5E, F). The cells cultured with

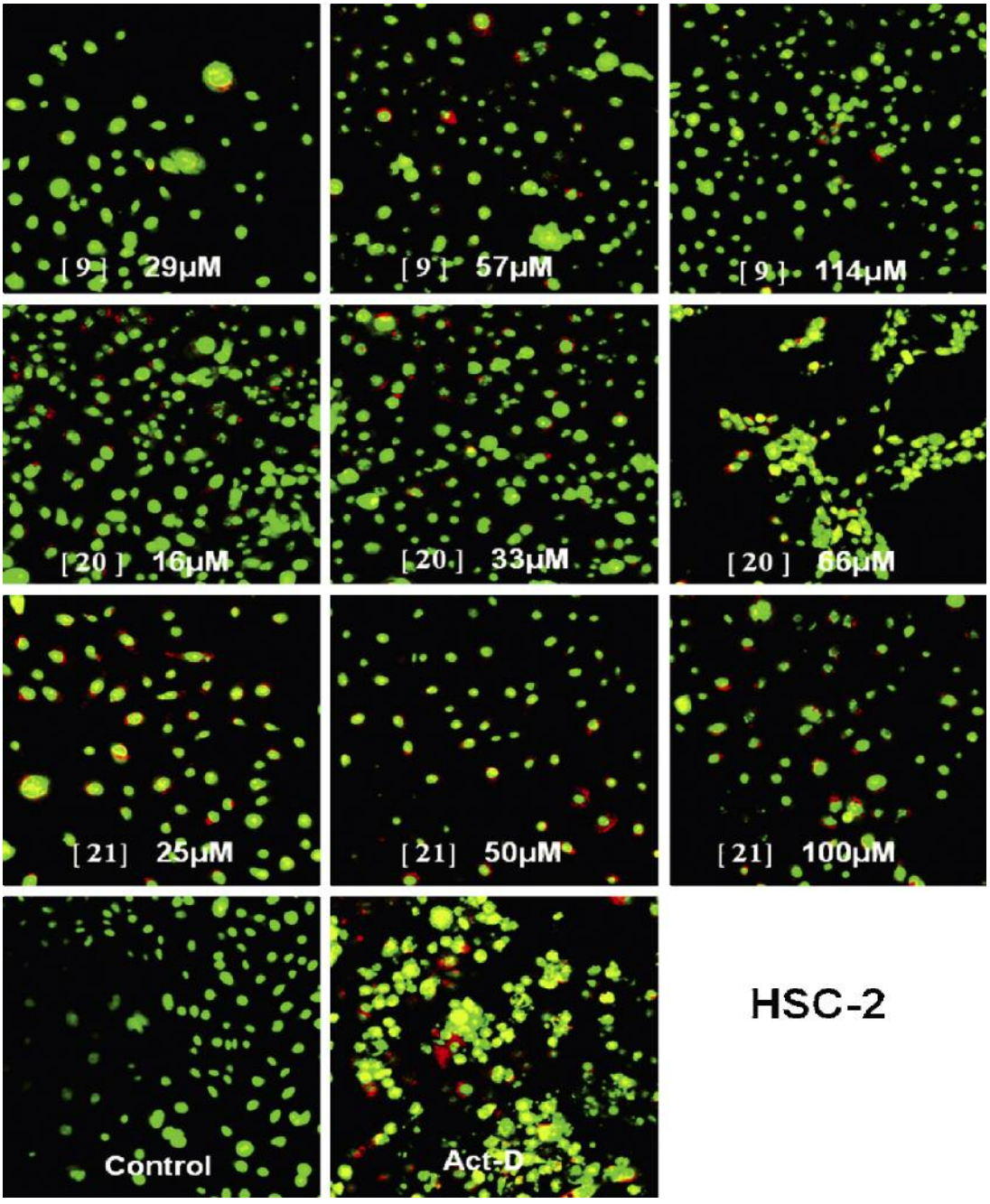

Figure 4. *Effect of benzocycloheptoxazine derivatives **[9, 20, 21]** on the cytoplasmic dsistribution of acridine orange. HSC-2 cells were incubated for 6 hours with the indicated concentrations of **[9]**, **[20]** or **[21]**. Excitation filter 488 nm, emission filter 505-530 nm (green) and >650 nm (red).*

compound **[21]** showed no evident changes at the CC_{50} (25 μM) or at twice the CC_{50} (50 μM) (Figure 5G, H). However, at four times the CC_{50} (100 μM), the vacuolation of the endoplasmic reticulum and the presence of a number of lamellar body-like organelles were found (Figure 5H, I).

Discussion

The present study demonstrates that parent compounds such as benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]**, benzo[*b*] cyclohepta[*e*][1,4]oxazine **[6]** and 14*H*-[1,4]benzoxazino [3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine **[24]** alone had very weak cytotoxicity and tumor-specificity. The introduction of three bromines to the cycloheptane ring of compounds **[1]**

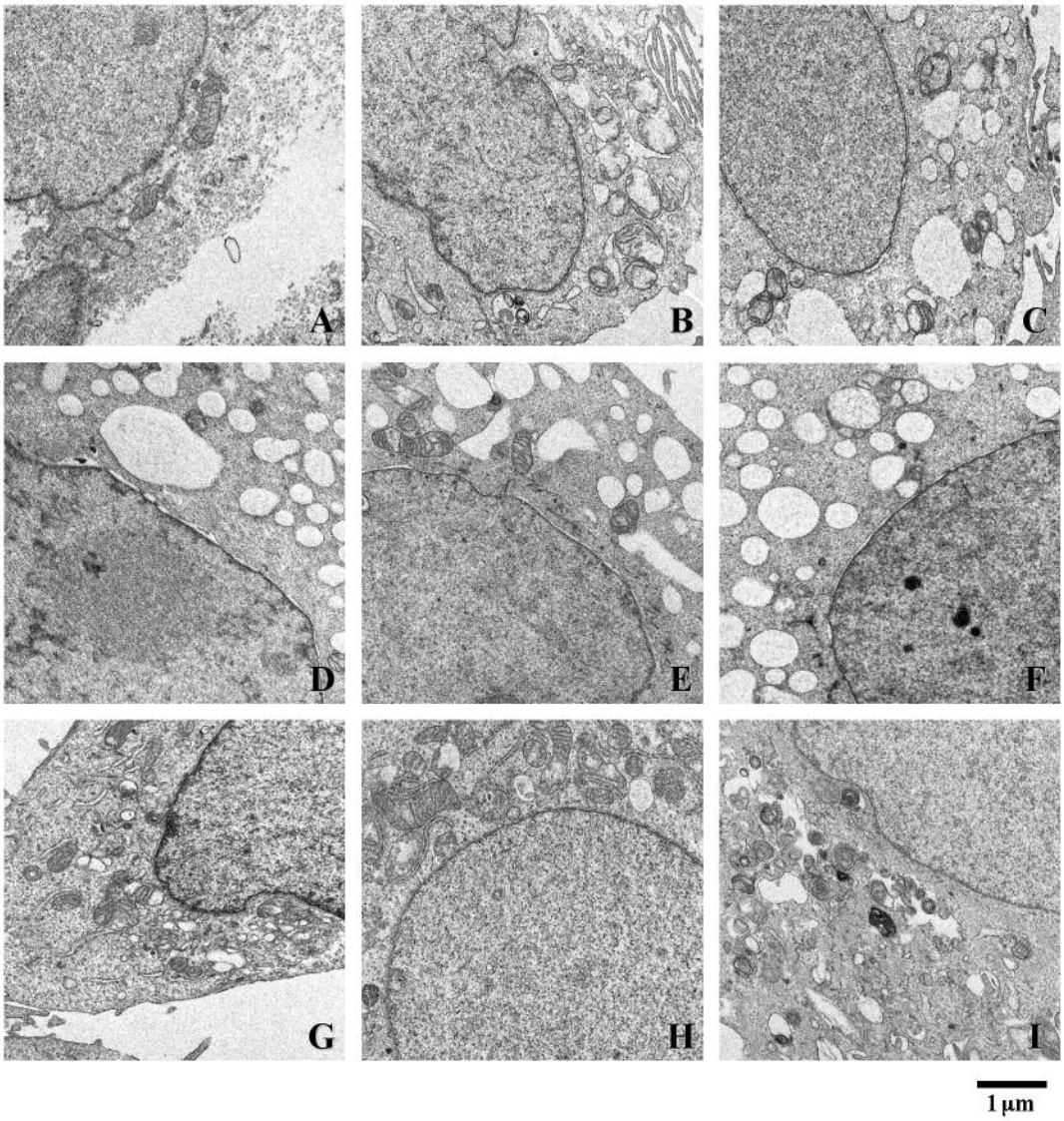

Figure 5. *Electron microscopy of control and dying HSC-2 cells. HSC-2 cells were incubated for 4 hours without treatment (control) (A) or with **[9]** at 29 (B), 57 (C) or 114 μM (D), **[20]** at 16 (E) or 66 μM (F), or **[21]** at 25 (G), 50 (H) or 100 μM (I) and then processed for electron microscopy. Bar: 1 μm.*

and **[6]** significantly enhanced their cytotoxicity against tumor cells, elevating the tumor-specificity index (TS=5.6, 12.5). The introduction of one bromine at C-6 of the cycloheptane ring **[7]** slightly increased the tumor-specific index (TS=2.6), but the introdution of bromine together with a methyl group **[20]** or chlorine **[21]** at C-2 of the benzene ring significantly enhanced the tumor-specificity (TS=9.1 and 11.5, respectively).

The present study also demonstrated that the most tumor-selective compounds **[9, 20, 21]** induced apoptotic cell death to various extents in the three tumor cell lines. By treatment with any of these compounds, HL-60 cells were committed to apoptotic cell death. On the other hand, HSC-2 cells were committed to autophagic cell death at lower concentrations and apoptotic cell death at higher concentrations, suggesting the interconvertibility between autophagy and apoptosis, depending on the concentration. This is consistent with recent reports that inhibitors of autophagy stimulated cell death toward apoptosis (32, 33).

We recently reported that 1-trichloroacetyl-3-bromo-2-methoxyazulene and 1-trichloroacetyl-3-chloro-2-ethoxyazulene induced apoptotic cell death in HL-60 cells, whereas they induced autophagic cell death in HSC-4 cells (34). Since HSC-4 cells are much more resistant to these compounds, the induction of autophagic cell death may be

related to the extent of drug resistance. This is supported by the fact that malignant brain tumor cell lines, which are resistant to many anticancer drugs, have been reported to undergo autophagy (35, 36).

In conclusion, we found that benzocycloheptoxazines derivatives induced different types of cell death depending on the type of cells and on the concentration used. Further study is required to clarify the mechanism by which such diversity is produced.

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References

1 Nozoe T, Takase K, Matsumura H, Asao T, Kikuchi K and Ito S: Non-benzenoid aromatic compounds. *In*: Dai-Yuki Kagaku, Kotake M (ed.). Tokyo, Asakura-Shoten Publ. Co., Vol. 13, pp. 1-656, 1960.

2 Nozoe T, Breslow R, Hafner K, Ito S and Murata I: Topics in nonbenzenoid aromatic chemistry. Tokyo, Hirokawa Publ. Co., Vol. I, pp. 1-295, 1973.

3 Nozoe T, Breslow R, Hafner K, Ito S and Murata I: Topics in nonbenzenoid aromatic chemistry. Tokyo, Hirokawa Publ Co, Vol. II, pp. 1-379, 1977.

4 Inamori Y, Shinohara S, Tsujibo H, Okabe T, Morita Y, Sakagami Y, Kumeda Y and Ishida N: Antimicrobial activity and metalloprotease inhibition of Hinokitiol-related compounds, the constituents of *Thujopsis dolabrata* S. and Z. *hondai* Mak. Biol Pharm Bull *22*: 990-993, 1999.

5 Inamori Y, Sakagami Y, Morita Y, Shibata M, Sugiura M, Kumeda Y, Okabe T, Tsujibo H and Ishida N: Antifungal activity of Hinokitiol-related compounds on wood-rotting fungi and their insecticidal activities. Biol Pharm Bull *23*: 995-997, 2000.

6 Inamori Y, Nishiguchi K, Matsuo N, Tsujibo H, Baba K and Ishida N: Phytogrowth-inhibitory activities of tropolone and hinokitiol. Chem Pharm Bull *39*: 2378-2381, 1991.

7 Sakagami Y, Inamori Y, Isoyama N, Tsujibo H, Okabe T, Morita Y and Ishida N: Phytogrowth-inhibitory activities of β-dolabrin and γ-thujaplicin, hinokitiol-related compounds and constituents of *Thujopsis dolabrata* Sieb. and Zucc. var *hondai* Makino. Biol Pharm Bull *23*: 645-648, 2000.

8 Inamori Y, Tsujibo H, Ohnishi H, Ishii F, Mizugaki M, Aso H and Ishida N: Cytotoxic effect of hinokitiol and tropolone on the growth of mammalian cells and on blastogenesis of mouse splenic T-cells. Biol Pharm Bull *16*: 521-523, 1993.

9 Matsumura E, Morita Y, Date T, Tsujibo H, Yasuda M, Okabe T, Ishida N and Inamori Y: Cytotoxicity of the hinokitiol-related compounds, γ-thujaplicin and β-dolabrin. Biol Pharm Bull *24*: 299-302, 2001.

10 Borchardt RT: Catechol *O*-methyltransferase. 1. Kinetices of tropolone inhibition. J Medicinal Chem *16*: 377-382, 1973.

11 Wakabayashi H, Yokoyama K, Hashiba K, Hashimoto K, Kikuchi H, Nishikawa H, Kurihara T, Satoh K, Shioda S, Muto S, Terakubo S, Nakashima H, Motohashi N and Sakagami H: Cytotoxic activity of tropolones against human oral tumor cell lines. Anticancer Res *23*: 4757-4764, 2003.

12 Yokoyama K, Hashiba K, Wakabayashi H, Hashimoto K, Satoh K, Kurihara T, Motohashi N and Sakagami H: Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by tropolones. Anticancer Res *24*: 3917-3922, 2004.

13 Nozoe T: Cyclohepta[*b*][1,4]benzoxazine and its related compounds. Some novel aspects in heterocyclic chemistry. Heterocycles *30*: 1263-1306, 1990.

14 Nozoe T, Asao T and Takahashi K: Synthesis of benzo[*b*] tropothiazine and its derivatives. Bull Chem Soc Jpn *34*: 146-150, 1961.

15 Shindo K, Ishikawa S and Nozoe T: Cyclohepta[*b*][1,4] benzothiazines and their diazine analogs. 1. Formation and reactions of cyclohepta[*b*][1,4]benzothiazines. Bull Chem Soc Jpn *58*: 165-171, 1985.

16 Nozoe T, Shindo K and Ishikawa S: Novel intermolecular heterocycle exchange reaction of cyclohepta[*b*][1,4]benzoxazines and their *S*-analogs with 1,2-bifunctional reagents. Chem Lett pp. 1593-1596, 1988.

17 Shindo K, Ishikawa S and Nozoe T: Cyclohepta[*b*][1,4] benzothiazines and their diazine analogs. 2. Formation and properties of cyclohepta[*b*]quinoxalines. Bull Chem Soc Jpn *62*: 1158-1166, 1989.

18 Kurihara T, Shindo K, Ishikawa S and Nozoe T: Benzo[*b*]cyclohepta[*e*][1,4]thiazines and their diazine analogs. III. π-Electronic structures of 5*H*,11*H*-cyclohepta[*b*] quinoxalinium ions and their *O* and *S* analogs. Bull Chem Soc Jpn *64*: 2668-2676, 1991.

19 Nozoe T, Kitahara Y, Takase K and Sasaki M: Syntheses of benzo[*b*]tropazines. Proc Japan Acad *32*: 349-352, 1954.

20 Nozoe T, Okai H and Someya T: Reactive troponoids and *o*-aminophenol. I. Synthesis of cyclohepta[*b*][1,4]benzoxazine. Bull Chem Soc Jpn *51*: 2185-2186, 1978.

21 Nozoe T and Someya T: Reactive troponoids and *o*-aminophenol. II. The formation of cyclohepta[*b*][1,4] benzoxazine and 11*H*-cyclohepta[*b*][1,4]benzoxazin-10-one derivatives from isomeric isopropyl-2-chlorotropones. Bull Chem Soc Jpn *51*: 3316-3319, 1978.

22 Nozoe T, Okai H, Wakabayashi H and Ishikawa S: Novel intermolecular heterocycle exchange reaction of cyclohepta[*b*] [1,4]benzoxazines with *o*-aminophenol derivatives. Chem Lett pp. 1589-1592, 1988.

23 Nozoe T, Someya T and Okai H: Reactive troponoids and o-aminophenol. III. The reaction of 2-bromo-7-methoxytropone. Bull Chem Soc Jpn *52*: 1156-1158, 1979.

24 Someya T, Okai H, Wakabayashi H and Nozoe T: Reactive troponoids and *o*-aminophenol. V. The reaction of 3-bromo-2-methoxytropone and *o*-aminophenol. Bull Chem Soc Jpn *56*: 2756-2761, 1983.

25 Nozoe T, Okai H, Wakabayashi H and Ishikawa S: A novel synthesis of cyclohepta[2,1-*b*:2,3-*b*']di[1,4]benzoxazines and 14*H*[1,4]benzoxazino[3',2':3,4]- cyclohepta[1,2-*b*][1,4]benzoxazines. Chem Lett pp. 1145-1148, 1984.

26 Wakabayashi H, Ishikawa S, Okai H and Nozoe T: Intermolecular bromine transfer of 2-amino-7-bromotropone derivatives catalyzed by strong acid. Bull Chem Soc Jpn *58*: 2840-2843, 1985.

27 Nozoe T, Okai H, Wakabayashi H and Ishikawa S: Cyclohepta [*b*][1,4]benzoxazines. 3. The reaction of 6-bromocyclohepta

[*b*][1,4]benzoxazine with o-aminophenol. Bull Chem Soc Jpn *62*: 2307-2314, 1989.

28 Nozoe T, Wakabayashi H and Ishikawa S: Reaction of 5-bromo-2-methoxytropone and 8-bromo- and 6,8-dibromo-cyclohepta [*b*][1,4]benzoxazines with *o*-aminophenol. Heterocycles *29*: 1005-1012, 1989.

29 Nozoe T, Shindo K, Wakabayashi H and Ishikawa S: Poly-annulated tropylium compounds having benzothiazine and related heterocyclic rings. Formation of *o*- and *p*-benzoquinonoid products by rearrangement. Heterocycles *29*: 1459-1464, 1989.

30 Wakabayashi H, Kurihara T, Ishikawa S, Okada J and Nozoe T: Benzo[*b*]cyclohept[*e*][1,4]oxazines 4. Synthesis and properties of all possible benzoxazinotropones. Bull Chem Soc Jpn *64*: 2131-2139, 1991.

31 Satoh R, Kishino K, Morshed SRM, Takayama F, Otsuki S, Suzuki F, Hashimoto K, Kikuchi H, Nishikawa H, Yasui T and Sakagami H: Change in fluoride sensitivity during *in vitro* senescence of human normal oral cells. Anticancer Res *25*: 2085-2090, 2005.

32 Cui Q, Tashiro S, Onodera S and Ikejima T: Augmentation of oridonin-induced apoptosis observed with reduced autophagy. J Pharmacol Sci *101*: 230-239, 2006.

33 Li D, Cui Q, Chen SG, Wu LJ, Tashiro S, Onodera S and Ikejima T: Inactivation of ras and changes of mitochondrial membrane potential contribute to oridonin-induced autophagy in a 431 cells. J Pharmacol Sci *105*: 22-33, 2007.

34 Sekine T, Takahashi J, Nishishiro M, Arai A, Wakabayashi H, Kurihara T, Kobayashi M, Hashimoto K, Kikuchi H, Katayama T, Kanda Y, Kunii S, Motohashi N and Sakagami H: Tumor specificity and type of cell death induced by trihaloacetylazulenes in human tumor cell lines. Anticancer Res *27*: 133-144, 2007.

35 Kanzawa T, Kondo Y, Ito H, Kondo S and Germano I: Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res *63*: 2103-2108, 2003.

36 Daido S, Yamamoto A, Fujisawa K, Sawaya R, Kondo S and Kondo Y: Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. Cancer Res *65*: 4368-4375, 2005.

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