

Apoptosis-inducing Activity of Trihaloacetylazulenes against Human Oral Tumor Cell Lines

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Abstract. Twenty-six trihaloacetylazulene derivatives were investigated for their tumor-specific cytotoxicity and apoptosis-inducing activity against three human normal cells (HGF, HPC, HPLF) and four human tumor cell lines (HSC-2, HSC-3, HSC-4, HL-60). The trichloroacetylazulenes [**1b-13b**] generally showed higher cytotoxicity as compared to the corresponding trifluoroacetylazulenes [**1a-13a**]. The trichloroacetylazulenes [**1b-13b**] also showed higher tumor-specific cytotoxicity (expressed as TS value) than the corresponding trifluoroacetylazulenes [**1a-13a**]. Especially, 2,3-dimethyl-1-trichloroacetylazulene [**5b**] and 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [**11b**] showed the highest cytotoxicity and tumor specificity (TS >35.6 and >44.1, respectively). These compounds induced internucleosomal DNA fragmentation in HL-60 cells, but not in HSC-2 and HSC-3 cells, but activated caspase-3, -8 and -9 in all of these cells, suggesting the activation of both mitochondria-independent (extrinsic) and -dependent (intrinsic) pathways. Western blot analysis showed that two compounds [**5b**, **11b**] slightly increased the intracellular concentration of pro-apoptotic proteins (Bad, Bax) in HSC-2 cells. None of the 26 compounds showed anti-HIV activity. These results suggest [**5b**] and [**11b**] as possible candidates for future cancer chemotherapy.

Azulene (1-4), an isomer of naphthalene, has a dipole moment and a resonance energy with intermediate values

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between those of benzene and naphthalene and is considerably more reactive, when compared with two arenes. Azulene derivatives have been investigated for their synthesis and chemical reactions (5-7). Their derivatives have shown several biological activities, including antibacterial activity (8), anti-ulcer activity (9), relaxant activity (10), inhibition of thromboxane A₂-induced vasoconstriction and thrombosis (11), acute toxicity and local anesthetic activity (12) and chemotherapeutic activity against mucous membrane diseases (13, 14). However, the effects of azulene derivatives on cellular function have not yet been investigated in detail.

We have recently initiated the study of the structure-activity relationship of a total of 81 azulene (15, 16), tropolone (17, 18) and azulenequinone (19, 20) derivatives. 1,3-Difluoroazulene (15), 2,4-dibromo-7-methoxytropone (17), 3-morpholino-1,5-azulenequinone and 3,7-dibromo-1,5-azulenequinone (19) were found to inhibit the NO production by activated mouse macrophages, without (15) or with (17, 19) the inhibition of iNOS mRNA and protein expression. In addition, 2-acetylaminiazulene, 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 a 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).

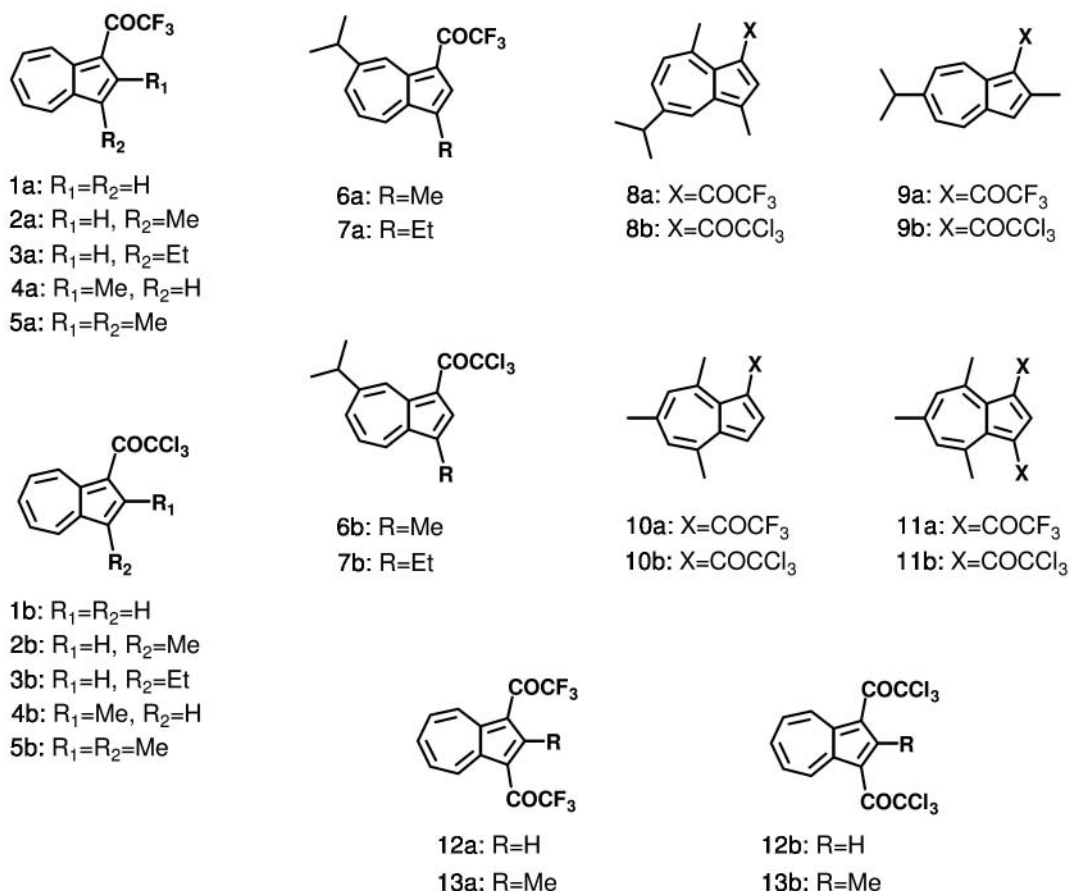


Figure 1. Structures of 13 trifluoroacetylazulenes [1a-13a] and 13 trichloroacetylazulenes [1b-13b].

Hexafluorotrihydroxyvitamin D₃ derivatives have shown higher differentiation-inducing activity (21, 22) than the parent compound, suggesting that the introduction of fluoride may have enhanced the biological activity of vitamin D₃. However, it is not clear whether the introduction of halogens other than fluoride show a similar enhancing effect. Whether trifluoroacetylazulenes [1a-13a] and trichloroacetylazulenes [1b-13b] display tumor-specific cytotoxic activity was investigated using 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)]. Whether such compounds induce apoptosis-associated characteristics (such as DNA fragmentation, caspase activation and apoptosis-related protein expression) in human tumor cells was also examined.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco'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) (Wako Pure Chem, Ind., Ltd., Osaka, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT), 3'-azido-2', 3'-dideoxythymidine (AZT), dideoxycytidine (ddC) (Sigma Chem Co., St. Louis, MO, USA).

Synthesis of trihaloacetylazulenes. The trihaloacetylazulene derivatives were synthesized according to the published methods for the following compounds: 1-trifluoroacetylazulene [1a] (23,24), 3-methyl-1-trifluoroacetylazulene [2a] (25), 3-ethyl-1-trifluoroacetylazulene [3a] (24-26), 2-methyl-1-trifluoroacetylazulene [4a] (25), 2,3-dimethyl-1-trifluoroacetylazulene [5a] (24-26), 7-isopropyl-3-methyl-1-trifluoroacetylazulene [6a] (24-26), 3-ethyl-7-isopropyl-1-trifluoroacetylazulene [7a] (24-26), 3-trifluoroacetylguaiazulene [8a] (26, 27), 6-isopropyl-2-methyl-1-trifluoroacetylazulene [9a] (24-26), 1-trifluoroacetyl-4,6,8-trimethylazulene [10a] (26, 27), 1,3-ditrifluoroacetyl-4, 6,8-trimethylazulene [11a] (28-30), 1,3-ditrifluoro acetylazulene [12a] (28-30), 2-methyl-1,3-ditrifluoroacetylazulene [13a] (28-30), 1-trichloroacetylazulene [1b] (24), 3-methyl-1-trichloroacetylazulene [2b] (24-26), 3-ethyl-1-trichloroacetylazulene [3b] (24-26), 2-methyl-1-trichloroacetylazulene [4b] (24-26), 2,3-dimethyl-1-trichloroacetylazulene [5b] (24-26), 7-isopropyl-3-methyl-1-trichloroacetylazulene [6b] (24-26), 3-ethyl-7-

Table I. Cytotoxic activity of trihaloacetylazulenes.

| Compd. | MW | Cytotoxic activity (CC ₅₀ : μ M) | | | | | | | TS |
|--------|-------|---|--------|--------|------------------------|--------|--------|-------|-------|
| | | Normal human cells | | | Human tumor cell lines | | | | |
| | | HGF | HPLF | HPC | HSC-2 | HSC-3 | HSC-4 | HL-60 | |
| [1a] | 224 | 159.8 | 161.9 | >191.7 | 92.5 | 106.3 | 129.7 | 59.6 | >1.7 |
| [2a] | 238 | >179.7 | >198.0 | >200.0 | 125.2 | >187.7 | 172.0 | 59.5 | ><1.4 |
| [3a] | 252 | >200.0 | >191.6 | >200.0 | 170.8 | >178.0 | >200.0 | 62.0 | ><1.3 |
| [4a] | 238 | 177.0 | 120.9 | >187.9 | 69.9 | 101.6 | 79.8 | 43.3 | >2.2 |
| [5a] | 252 | >200.0 | >200.0 | >200.0 | >200.0 | 153.5 | >176.5 | 31.7 | ><1.7 |
| [6a] | 280 | >200.0 | >200.0 | >200.0 | >200.0 | >161.0 | >146.4 | 31.8 | ><1.8 |
| [7a] | 294 | >200.0 | >200.0 | >200.0 | 180.5 | 142.1 | 137.7 | 54.9 | >1.8 |
| [8a] | 294 | >200.0 | >200.0 | >200.0 | >200.0 | 128.0 | >135.7 | 69.9 | ><1.8 |
| [9a] | 280 | >200.0 | >200.0 | >200.0 | >200.0 | >174.9 | >197.2 | 40.6 | ><1.5 |
| [10a] | 266 | >200.0 | >200.0 | >200.0 | >200.0 | >200.0 | >200.0 | 27.5 | ><1.4 |
| [11a] | 362 | >200.0 | >200.0 | >200.0 | >200.0 | 140.2 | >200.0 | 20.4 | ><1.7 |
| [12a] | 320 | >200.0 | 199.0 | >200.0 | 168.2 | 139.6 | 68.5 | 198.1 | >1.5 |
| [13a] | 334 | 165.3 | 190.3 | >200.0 | 151.2 | 167.2 | 130.6 | 38.6 | >1.7 |
| [1b] | 273.5 | 159.3 | 95.1 | 98.6 | 36.5 | 40.4 | 34.0 | 16.5 | 3.9 |
| [2b] | 287.5 | >167.9 | >200.0 | >200.0 | 106.4 | 133.7 | 103.5 | 5.4 | >2.3 |
| [3b] | 301.5 | >200.0 | >200.0 | >200.0 | 140.5 | 115.5 | >200.0 | 26.4 | ><1.8 |
| [4b] | 287.5 | <107.7 | 152.6 | 138.7 | 3.6 | 36.5 | 3.9 | 1.6 | <9.5 |
| [5b] | 301.5 | 158.4 | 115.8 | 108.6 | <1.6 | 7.3 | 1.8 | 1.6 | >35.6 |
| [6b] | 329.5 | >200.0 | >200.0 | >200.0 | >131.9 | 65.3 | >123.2 | 7.6 | ><3.1 |
| [7b] | 343.5 | 199.5 | >200.0 | >200.0 | 120.0 | 65.0 | 68.0 | 18.8 | >3.9 |
| [8b] | 343.5 | 47.1 | 19.6 | 36.6 | 8.4 | 6.5 | 10.7 | 9.7 | 3.8 |
| [9b] | 329.5 | 96.4 | 104.4 | 169.8 | 23.4 | 34.9 | 29.3 | 14.8 | 4.7 |
| [10b] | 315.5 | >200.0 | >192.3 | >200.0 | 27.3 | 9.2 | 16.0 | 4.9 | >19.6 |
| [11b] | 461 | 153.2 | >172.9 | >200.0 | 29.0 | <6.1 | 3.5 | 2.3 | >44.1 |
| [12b] | 419 | >200.0 | >199.7 | >200.0 | 57.5 | 92.1 | 52.9 | 13.1 | >3.8 |
| [13b] | 433 | 160.7 | 176.7 | 177.0 | 52.4 | 8.9 | 40.7 | 1.9 | 10.0 |

TS: tumor-specific cytotoxicity. Each value represents the mean from three to four independent experiments.

isopropyl-1-trichloroacetylazulene [7b] (24-26), 3-trichloroacetyl-guaiazulene [8b] (26), 6-isopropyl-2-methyl-1-trichloroacetylazulene [9b] (24-26), 1-trichloroacetyl-4,6,8-trimethylazulene [10b] (26), 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b] (28-30), 1,3-ditrichloroacetylazulene [12b] (28-30) and 2-methyl-1,3-ditrichloroacetylazulene [13b] (28-30).

Cell culture. Three human oral tumor cell lines (HSC-2, HSC-3, HSC-4) 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% heat-inactivated FBS. The tumor cell lines were obtained from the Riken Cell Bank. Normal cells were prepared from periodontal tissues, according to the guidelines of Meikai University Ethics Committee (No. 0206), after obtaining informed consent from the patients.

Assay for cytotoxic activity. The cells (other than HL-60 cells) were inoculated at 5×10^3 cells/well in 96-microwell plates (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 h, the medium was removed by suction with an aspirator and replaced with 0.1 mL of fresh medium containing various concentrations of the test compounds. The first well contained 200 μ M sample which

was diluted two-fold sequentially, with three replicate wells for each concentration. The cells were incubated for another 24 h and the relative viable cell number was then determined by the MTT method. In brief, fresh culture medium containing 0.2 mg/mL MTT was added to the cells which were then incubated for another 4 h. The cells were lysed with 0.1 mL of DMSO and the absorbance of the cell lysate at 540 nm was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland) (16, 18, 20). The A₅₄₀ of the control cells were usually in the range from 0.40 to 0.90.

The HL-60 cells were inoculated at $7 \times 10^4/0.1$ mL in 96-microwell plates to which were added various concentrations of the test compounds. After incubation for 24 h, the viable cell number was determined by trypan blue exclusion under a light microscope.

The 50% cytotoxic concentration (CC₅₀) was determined from the dose-response curve. Tumor specificity (TS) was determined by the following equation: $TS = \{ [CC_{50} (HGF) + CC_{50} (HPC) + CC_{50} (HPLF)] / [CC_{50} (HSC-2) + CC_{50} (HSC-3) + CC_{50} (HSC-4) + CC_{50} (HL-60)] \} \times (4/3)$

Assay for DNA fragmentation. The cells were washed once with PBS (-) and lysed with 50 μ L lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium N-lauroylsarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 h at 50 °C, and then mixed with 50 μ L NaI

solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], followed by 250 μ L of ethanol. After centrifugation for 20 min 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). The sample (10-20 μ L) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells, induced by UV irradiation, were run in parallel (16, 18, 20). After staining with ethidium bromide, DNA was visualized by UV irradiation and photographed with a CCD camera (Bio Doc Inc., UVP).

Assay for caspase activation. The cells were washed with PBS and lysed in the lysis solution (MBL, Nagoya, Japan). After standing for 10 min on ice followed by centrifugation for 5 min at 10,000 xg, the supernatant was collected. The lysate (50 μ L, equivalent to 200 μ g protein) was mixed with 50 μ L 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 h at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by a plate reader (16, 18, 20).

Western blotting. The cells were lysed with 100 μ L of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 min in ice water and were then incubated for 50 min at 4°C with RT-5 ROTATOR (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 min at 4°C to remove insoluble materials and the supernatant was collected. The protein concentration of the supernatant was determined by a Protein Assay Kit (Bio Rad, Hercules, CA, USA). The cell lysates (containing 15 μ g protein) were mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol), boiled for 10 min, applied to SDS-12% polyacrylamide gel electrophoresis and were then transferred to the polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in PBS plus 0.05% Tween 20 overnight at 4°C and incubated with anti-Bcl-2 antibody (1:1,000), anti-Bax antibody (1:1,000), or anti-actin antibody (1:1,000) (Santa Cruz Biotechnology, Delaware, CA, USA) for 90 min at room temperature and were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Immunoblots were developed by Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) and were analyzed on a Macintosh (Power Macintosh 7600/120) computer, using the public domain NIH Image Program (16, 18, 20).

Results

Structure and activity relationship. The trifluoroacetylazulenes [1a-13a] showed lower cytotoxicity compared to the trichloroacetylazulenes [1b-13b] (Table I). The relative cytotoxicity of 13 trifluoroacetylazulenes was comparable with that of the corresponding trichloroacetylazulenes. Especially trifluoroacetylazulenes [5a, 6a, 8a, 9a, 10a, 11a] showed much lower cytotoxic activity, whereas 3-trichloroacetylguaiazulene [8b] and 6-isopropyl-2-methyl-1-trichloroacetylazulene [9b] showed higher cytotoxicity for both

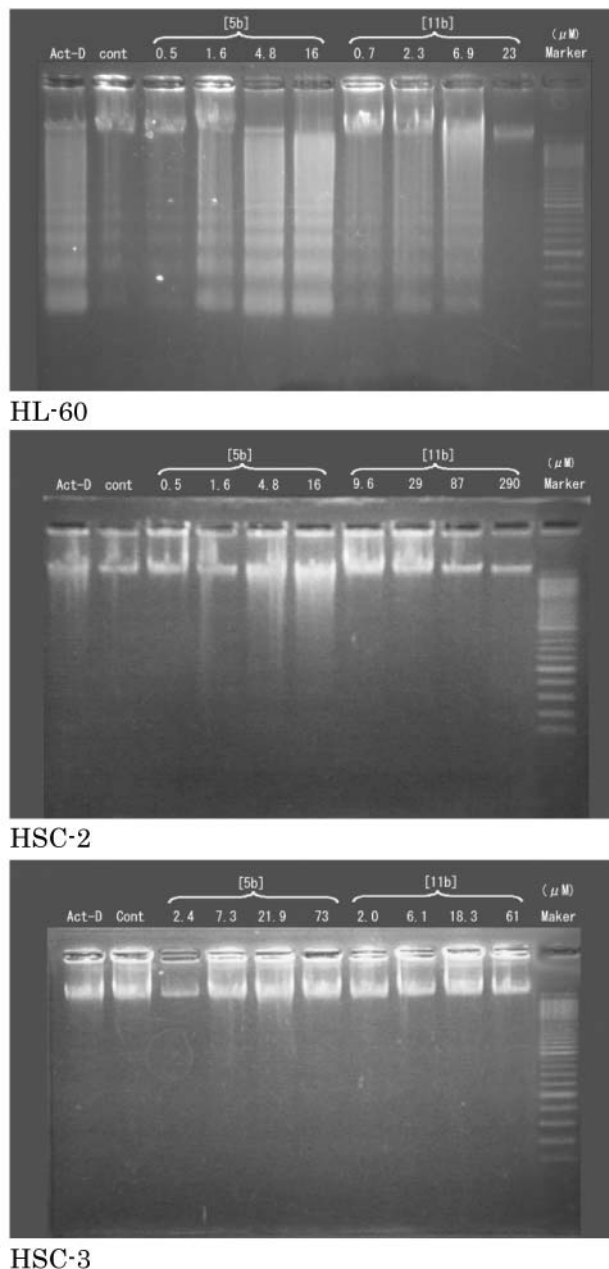


Figure 2. Induction of DNA fragmentation by two trichloroacetylazulenes. Near confluent HL-60, HSC-2 or HSC-3 cells were incubated for 6 h with the indicated concentrations of 2,3-dimethyl-1-trichloroacetylazulene [5b], 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b] or 1 μ g/mL actinomycin D (Act-D) (positive control). DNA was then extracted and applied to agarose gel electrophoresis. Marker DNA was run in lane 11. Representative data from three independent experiments are shown.

normal and tumor cells. In general, the trichloroacetylazulenes showed higher cytotoxicity than tropolone (18) and the azulenequinones (20). The trifluoroacetylazulenes generally showed lower cytotoxic activity than the corresponding azulenes (16). 2,3-Dimethyl-1-trichloro-

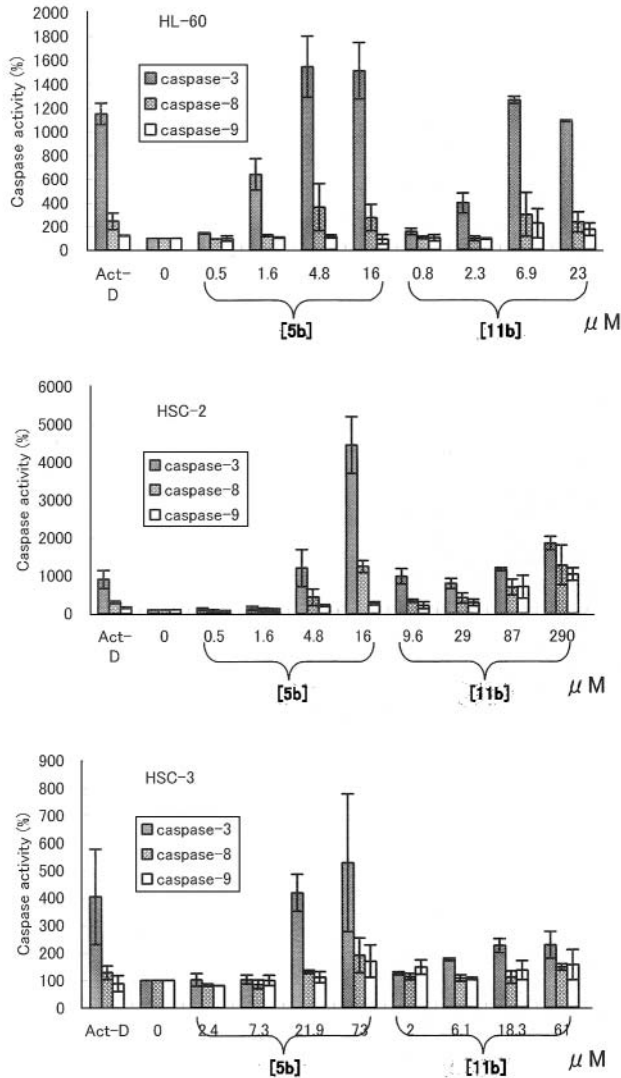


Figure 3. Activation of caspases-3, -8 and -9 by 2 trichloroacetylazulenes. HL-60 (upper panel), HSC-2 (center panel) and HSC-3 (lower panel) cells were incubated for 4 h without (control) or with the indicated concentrations of 2,3-dimethyl-1-trichloroacetylazulene [5b] or 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b] or 1 $\mu\text{g}/\text{mL}$ actinomycin D (Act-D) (positive control). The absorbance at 405 nm (the relative amount of the pNA released from the substrate for caspase-3, -8 and -9) in control, untreated cells was 0.047, 0.029, 0.014 (HL-60), 0.0085, 0.0055, 0.0040 (HSC-2) and 0.015, 0.018, 0.0105 (HSC-3), respectively. Each point represents mean \pm S.D. from 3 ~ 4 independent experiments.

acetylazulene [5b] and 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b] more efficiently killed the tumor cell lines than normal cells, yielding the highest tumor-specific cytotoxicity indices (TS) of >35.6 and >44.1 , respectively. 2-Methyl-1-trichloroacetylazulene [4b] (TS >9.5), 1-trichloroacetyl-4,6,8-trimethylazulene [10b] (TS >19.6) and 2-methyl-1,3-ditrifluoroacetylazulene [13b] (TS=10.0) showed slightly lower, but still high TS values.

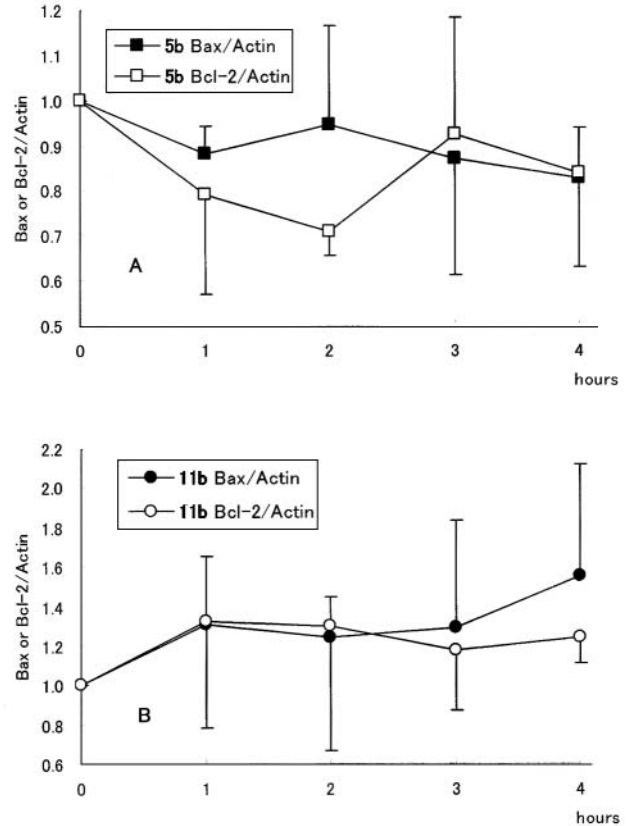


Figure 4. Effect of two trichloroacetylazulenes on the intracellular concentrations of apoptosis-related proteins. HL-60 cells were incubated for the indicated times without (control) or with 1.6 μM 2,3-dimethyl-1-trichloroacetylazulene [5b], 2.3 μM 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b]. The cell lysate (equivalent to 15 $\mu\text{g}/\text{mL}$) was subjected to Western blot analysis with respective antibodies. The intracellular concentration of Bax and Bcl-2 was quantified and expressed as the ratio to that of Actin and the control level of these proteins was normalized to 1.0. Each value represents mean \pm S.D. from three to four independent experiments.

Apoptosis induction. 2,3-Dimethyl-1-trichloroacetylazulene [5b] (higher than 1.6 μM) and 1,3-ditrichloroacetyl-4,6,8-trimethylazulene [11b] (higher than 2.3 μM) induced internucleosomal DNA fragmentation in HL-60 cells. Their effects reached maximal levels at 4.8 and 6.9 μM , respectively (Figure 2). They did not induce internucleosomal DNA fragmentation, but induced the production of large DNA fragments in the HSC-2 and HSC-3 cells (center and lower panels, Figure 2). Compound [5b] produced a smear pattern of DNA fragmentation, showing slightly higher DNA fragmentation activity than that of [11b].

Compounds [5b and 11b] activated caspase-3, -8 and -9 in all of these cells (Figure 3), suggesting the activation of both mitochondria-independent (extrinsic) and -dependent (intrinsic) pathways. The apoptosis-inducing activity of [5a] was slightly higher than that of [11b].

Western blot analysis showed that **[5b]** and **[11b]** did not significantly change the intracellular concentration of either pro-apoptotic (Bax) or anti-apoptotic (Bcl-2) proteins during the apoptosis induction of HL-60 cells (Figure 4).

Discussion

The results of the present study demonstrated that all 13 trichloroacetylazulenes showed higher cytotoxic activity against tumor cell lines than the corresponding trifluoroacetylazulenes. Whether the chlorination of other antitumor compounds results in a similar enhancement of cytotoxic activity remains to be investigated. Among the 26 trihaloacetylazulenes, 2,3-dimethyl-1-trichloroacetylazulene **[5b]** and 1,3-ditrichloroacetyl-4,6,8-trimethylazulene **[11b]** showed the highest tumor-specific cytotoxic activity (TS=>35.6, >44.1). This value is relatively high, since a total of 350 compounds including flavones, flavonols (3-hydroxyflavones) and isoprenoid-substituted flavonoids, benzophenones, xanthenes, anthraquinones, phenylbutanone glucoside, stilbene glucoside, isoflavones, isoflavanones, stilbenes (TS=0.3-31.7), coumarin derivatives (TS=1.0->11.0), procyanidines and flavonoids (TS=1.0->7.4), hydrolysable tannins (TS=1.0-8.2), triterpene aglycones and glycosides (TS=0.65->2.8), cycloartane glycosides and chromones (TS=0.7-1.4), furostane glycosides (TS=0.4->17.0), α,β -unsaturated ketones (TS=0.6-1.9), hydroxyketones (TS=1.0->17.6), β -diketones (TS=0.3-6.3), styrylchromones (TS=1.4-27.3), dihydroisoxazole and isoxazole derivatives (TS=0.9-1.6) showed lower tumor-specificity (31). We have recently reported that apoptosis-inducing agents (β -diketones (32), α,β -unsaturated ketones (33), hydroxyketones (34)) do not always show tumor-specific cytotoxicity and compounds with higher tumor specificity do not always induce apoptosis. Compounds **[5b]** and **[11b]** are unique in the sense that they have both higher tumor specificity and apoptosis-inducing activity.

It was found that **[5b]** and **[11b]** induced DNA fragmentation and activated both the mitochondria-independent extrinsic pathway (involved with caspase-8) and the mitochondria-dependent intrinsic pathway (involved with caspase-9) (35). It was unexpected that these compounds would induce little or no detectable change in the expressions of Bax and Bcl-2 proteins, since the pro-apoptotic Bax protein is usually up-regulated, whereas the anti-apoptotic Bcl-2 protein is down-regulated during apoptosis (35). The ratio of Bax/Bcl-2 determines the fate of cells, either directing them to survival or death. Additional study is required to explain these unexpected findings regarding the Bax and Bcl-2 expressions and to elucidate the killing mechanism.

We have investigated various biological activities of trihaloacetylazulenes **[1a-13a, 1b-13b]** and found that none

of these compounds showed anti-HIV activity (SI<1), in contrast to the potent anti-HIV activity of AZT (SI=6367) and ddC (SI=808), positive control (data not shown). Although the higher tumor specificity and apoptosis-inducing activity of **[5b]** and **[11b]** have been demonstrated, *in vivo* studies are required to evaluate their antitumor potential.

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