ANTICANCER RESEARCH *25*: 305-312 (2005)

Cytotoxic Activity of Azulenequinones Against Human Oral Tumor Cell Lines

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Abstract. *We investigated twenty-seven azulenequinone derivatives for their relative cytotoxicity against three human normal cell lines (HGF, HPC, HPLF) and four human tumor cell lines (HSG, HSC-2, HSC-3, HL-60). Parent 1,5-azulenequinone showed potent and some tumor-specific cytotoxicity. Halogenated derivatives of 1,5- and 1,7-azulenequinone showed potent cytotoxicity, but lower tumor-specific cytotoxicity. In contrast to other azulenequinones, amino derivatives such as 3-amino-1,5- and 1,7-azulenequinones showed relatively lower cytotoxic activity. The 3-Phenoxy-1,5-azuleneqinone derivative showed higher cytotoxicity than the 3-phenoxy-1,7-azulenequinone derivative. 1,5- and 1,7-Azulenequinones generally showed higher cytotoxicity, as compared with tropolones and azulene derivatives. 3-(3-Guaiazulenyl)-1,5-azulenequinone **[12]** and 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone **[24]** showed a relatively higher TS value and induced apoptosis (internucleosomal DNA fragmentation, activation of caspases 3, 8 and 9) in HL-60 and HSC-2 cells, possibly via the activation of both mitochondria-independent (extrinsic) and -dependent (intrinsic) pathways. Western blot analysis showed that **[24]** slightly increased the intracellular concentration of pro-apoptotic proteins (Bad, Bax) in HSC-2 cells, whereas **[12]** was much less active. None of the twenty-seven azulenequinones showed anti-HIV activity. These results suggest **[12]** and **[24]** as possible candidates for future cancer chemotherapy.*

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Key Words: Azulenequinones, apoptosis, caspase, DNA fragmentation, apoptosis-related protein expression.

Azulene derivatives (1-4) 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), and possible chemotherapeutic activity for mucous membrane diseases (10, 11). However, the effects of azulene derivatives on cellular function have not been investigated in detail. We have recently reported that methyl 7-isopropyl-2-methoxyazulene-1-carboxylate induced tumor-specific cytotoxicity and apoptotic cell death (characterized by internucleosomal DNA fragmentation and caspase 3 activation) in HL-60 cells, without involvement of the radical-mediated oxidation mechanism (12).

On the other hand, azulenequinone is a nonbenzenoid aromatic quinone (13, 14) and also an isomer of naphthoquinone. Naphthoquinones have shown antifungal, antibiotic, antimalarial, or antitumor activity (15).

We investigated, here, whether a total of twenty-seven azulenequinone derivatives (structure shown in Figure 1) display tumor-specific cytotoxic activity, using three normal human cell lines [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] and four human tumor cell lines [submandibular gland carcinoma (HSG), oral squamous cell carcinoma (HSC-2, HSC-3), promyelocytic leukemia (HL-60)], and, if so, whether azulenequinones induce apoptosis-associated characteristics (such as DNA fragmentation and caspase activation) in human tumor cells.

Materials and Methods

Methods. The following chemicals and reagents were obtained from the indicated companies: Dulbeccos's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS)(JRH, Bioscience, Lenexa, KS, USA); dimethyl sulfoxide (DMSO), diethylenetriaminepentaacetic acid

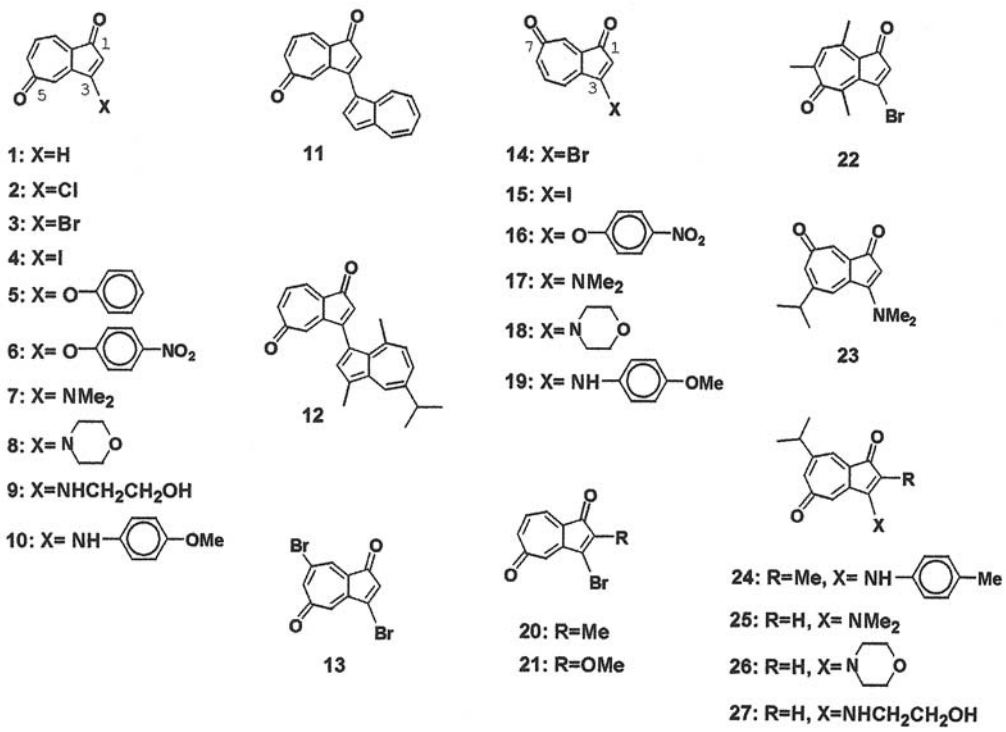

Figure 1. *Structure of azulenequinone derivatives.*

(DETAPAC) (Wako Pure Chem, Ind, Ltd, Osaka, Japan); RPMI 1640, 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 azulenequinone derivatives. Azulenequinone derivatives were synthesized, according to the published reports: 1,5-azulenequinone **[1]** (16-21), 3-chloro-1,5-azulenequinone **[2]** (19,21,22), 3-bromo-1,5-azulenequinone **[3]** (19,21,23), 3-iodo-1,5-azulenequinone **[4]** (19,21,22), 3-phenoxy-1,5-azulenequinone **[5]** (19,24), 3-(4-nitrophenoxy)-1,5-azulenequinone **[6]** (19,24), 3-(*N,N*-dimethylamino)-1,5-azulenequinone **[7]** (19,24), 3-morpholino-1,5-azulenequinone **[8]** (19,24) 3-(2-hydroxyethylamino)-1,5-azulenequinone **[9]** (19,24) 3-(4-methoxyanilino)-1,5-azulenequinone **[10]** (19,24), 3-(1-azulenyl)-1,5-azulenequinone **[11]** (19,24), 3-(3-guaiazulenyl)-1,5-azulenequinone **[12]** (19,24), 3,7-dibromo-1,5-azulenequinone **[13]** (19,21,23), 3-bromo-1,7-azulenequinone **[14]** (19,21,23), 3-iodo-1,7-azulenequinone **[15]** (19,21,22), 3-(4-nitrophenoxy)-1,7-azulenequinone **[16]** (19,24), 3-(*N,N*-dimethylamino)-1,7-azulenequinone **[17]** (19,24), 3-morpholino-1,7-azulenequinone **[18]** (19,24), 3-(4-methoxyanilino)-1,7-azulenequinone **[19]** (19,24), 3-bromo-2-methyl-1,5-azulenequinone **[20]** (19,20), 3-bromo-2-methoxy-1,5-azulenequinone **[21]** (19,25), 3-bromo-4,6,8-trimethyl-1,5-azulenequinone **[22]** (19,26), 3-(*N,N*-dimethylamino)-5-isopropyl-1,7-azulenequinone **[23]** (19), 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone **[24]** (19), 7-isopropyl-3-(*N,N*-dimethylamino)-1,5-azulenequinone **[25]** (19), 7-isopropyl-3-morpholino-1,5-azulenequinone **[26]** (19,27), 3-(2-hydroxyethylamino)-7-isopropyl-1,5-azulenequinone **[27]** (19,27).

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% heat-inactivated FBS. Tumor cell lines were obtained from the Riken Cell Bank. Normal cells were prepared from periodontal tissues, according to the guideline, of Meikai University Ethic Committee, Japan, (No. 0206) after obtaining informed consent from the patients.

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) (28). A_{540} of control cells were usually in the range from 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:

$$TS = \frac{CC_{50}(HGF) + CC_{50}(HPC) + CC_{50}(HPLF)}{CC_{50}(HSG) + CC_{50}(HSC\text{-}2) + CC_{50}(HSC\text{-}3) + CC_{50}(HL\text{-}60)} \times \frac{4}{3}$$

Table I. *Cytotoxic activity of azulenequinones.*

		Cytotoxic activity (CC_{50}:μM)							
		Normal human cells			Human tumor cell lines				
Compd.	MW	HGF	HPC	HPLF	HSG	HSC-2	HSC-3	HL-60	TS
1	158.16	30.6	37.3	25.5	8.9	<6.2	17.2	<3.9	>3.4
2	192.6	10.9	13.8	4.0	4.7	<4.0	18.0	<3.9	>1.3
3	237.05	54.4	22.0	15.3	17.9	<17.6	19.7	<3.9	>2.1
4	284.05	<9.1	17.8	12.1	6.0	6.7	7.8	<4.9	><2.1
5	250.26	127.0	29.0	23.5	11.3	2 .2	10.6	<4.2	>8.5
6	295.25	90.4	40.8	22.2	14.5	<3.9	21.8	<5.7	>4.5
7	201.25	406.0	294.0	>500.0	145.0	58.8	162.9	103.5	>3.4
8	243.26	114.0	314.0	>500.0	173.3	22.3	74.8	102.0	>3.3
9	217.22	>500.0	>500.0	>500.0	>500.0	52.3	>500.0	>500.0	><1.3
10	279.3	N.D.	275.0	200.0	90.0	27.2	200.0	>452.0	1.2
11	284.31	243.0	>500.0	>500.0	>500.0	117.0	309.4	40.5	><1.7
12	354.45	51.6	500.0	93.8	47.0	117.0	50.3	7.5	3.9
13	315.95	51.3	61.6	53.3	35.0	21.3	52.2	<3.9	>2.0
14	237.05	45.2	52.6	52.4	20.5	33.2	39.2	<4.0	>2.1
15	284.05	64.3	30.8	26.5	15.5	36.2	25.4	<4.9	>2.0
16	295.25	20.3	250.0	130.0	89.2	69.8	131.8	<26.0	>1.7
17	201.23	359.0	>500.0	>500.0	>500.0	347.0	>500.0	>497.5	><1.0
18	243.26	241.0	>500.0	>500.0	>500.0	232.0	456.0	>372.0	><1.1
19	279.3	214.0	500.0	188.0	>500.0	15.4	40.6	>439.0	<1.2
20	251.08	10.2	21.8	19.6	15.2	22.1	25.1	<4.4	>1.0
21	267.08	5.5	14.0	9.8	6.0	10.9	16.9	<3.7	>1.0
22	279.13	26.0	37.0	41.2	21.9	28.4	36.9	5.9	1.5
23	243.3	95.4	487.0	400.0	370.0	289.0	311.6	306.0	1.0
24	319.4	104.0	500.0	500.0	57.1	6.5	35.2	46.0	10.2
25	243.3	63.7	327.0	410.0	128.4	112.0	230.2	121.0	1.8
26	285.34	304.0	201.0	300.0	69.3	21.6	65.7	50.0	5.2
27	259.3	111.0	>500.0	471.0	293.8	207.0	324.9	244.0	>1.3

N.D., not determined due to the coloration.

The viability of HL-60 cells was determined by trypan blue exclusion, under a light microscope. HL-60 cells were inoculated at 5 x 10^4/0.1 mL in 96-microwell, and various concentrations of test compounds added. After incubation for 24 hours, the viable cell number was determined as described above. 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 lysate buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate), and incubated for 2 hours at 50˚C with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K (29). DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1), and precipitated with ethanol. DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). A DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by actinomycin D (1μg/mL) were run in parallel (29). After staining with ethidium bromide, DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc Inc, UVP).

Assay for caspase activation. Cells were washed with PBS and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 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 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.

Western blotting. The cells were lysed with 100 μL 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 minutes in ice water, and then incubated for 50 minutes at 4˚C with RT-5 ROTATOR (Titec, Saitama, Japan). Cell lysates were centrifuged at 16,000 xg for 20 minutes at 4˚C to remove insoluble materials and the supernatant was collected. The protein concentration of the supernatant was determined by Protein Assay Kit (Bio Rad, Hercules, CA, USA). Cell lysates (containing 15 μg 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% bromphenol blue, 1.2% 2-mercaptoethanol), boiled for 10 minutes, and applied to SDS-12% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The mebranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) plus 0.05% Tween 20 overnight at 4˚C and incubated with anti-

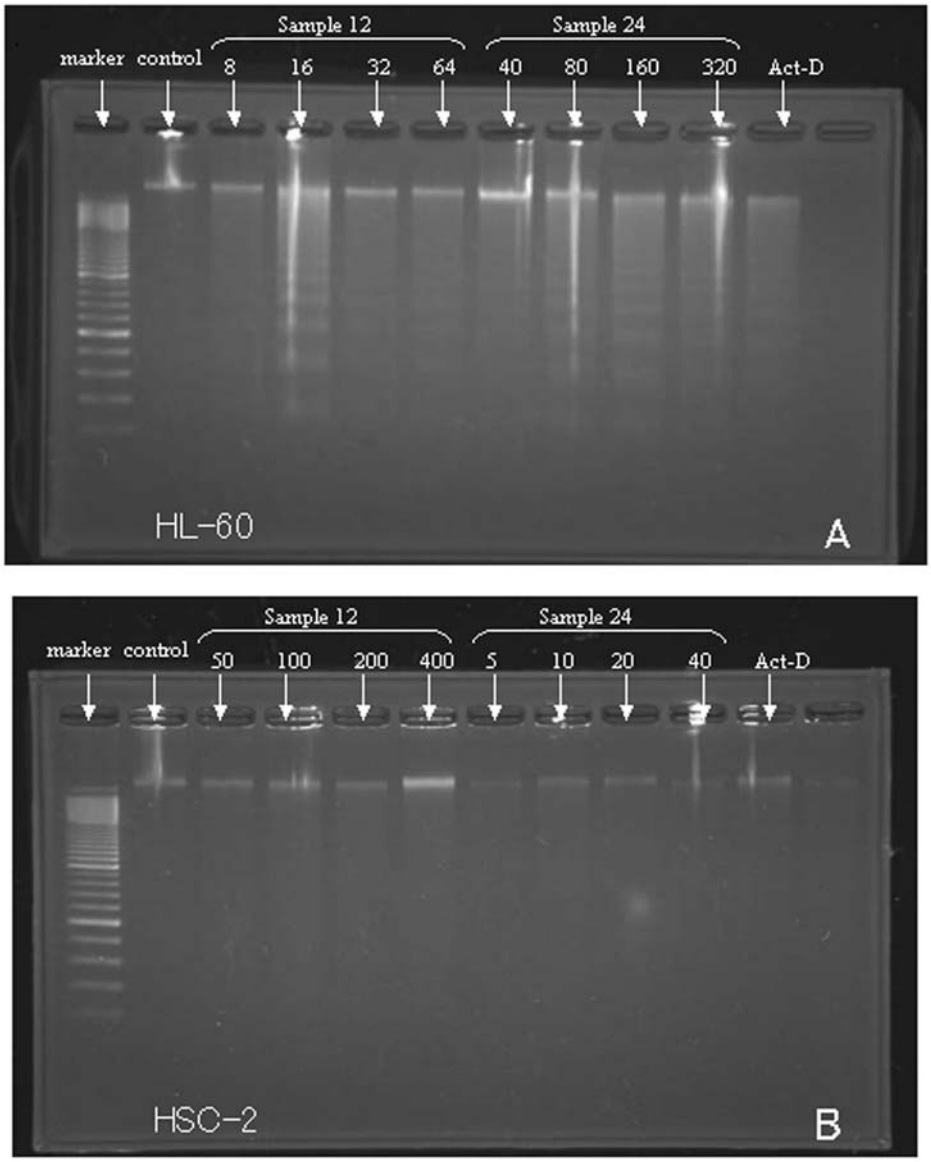

Figure 2. *Induction of DNA fragmentation by* ***[12]*** *and* ***[24]****. Near confluent HL-60 (A) and HSC-2 (B) cells were incubated for 6 hours with the indicated concentrations of 3-(3-guaiazulenyl)-1,5-azulenequinone* ***[12]****, 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone* ***[24]*** *or with 1 μg/mL actinomycin D (Act-D). DNA was then extracted and applied to agarose gel electrophoresis. Marker DNA was run in lane 1.*

Bcl-2 antibody (1:1,000), anti-Bax antibody (1:1,000), anti-Bad antibody (1:1,000) (Santa Cruz Biotechnology, Delaware, CA, USA) or anti-actin antibody (1:1,000) (Sigma) for 90 minutes at room temperature, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature. Immunoblots were developed by Western Lightning™ Chemiluminescence Reagent *Plus* (Perkin Elmer Life Sciences, Boston, MA, USA), and analyzed on a Macintosh (Power Macintosh 7600/120) computer, using the public domain NIH Image Program (developed at the U.S. National Institute of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, USA, part number PB95-500195GEI).

Assay for anti-human immunodeficiency virus (HIV) activity. MT-4 cells were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock- infected (control) MT-4 cells (1.5×10^5/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 (30). 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:

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

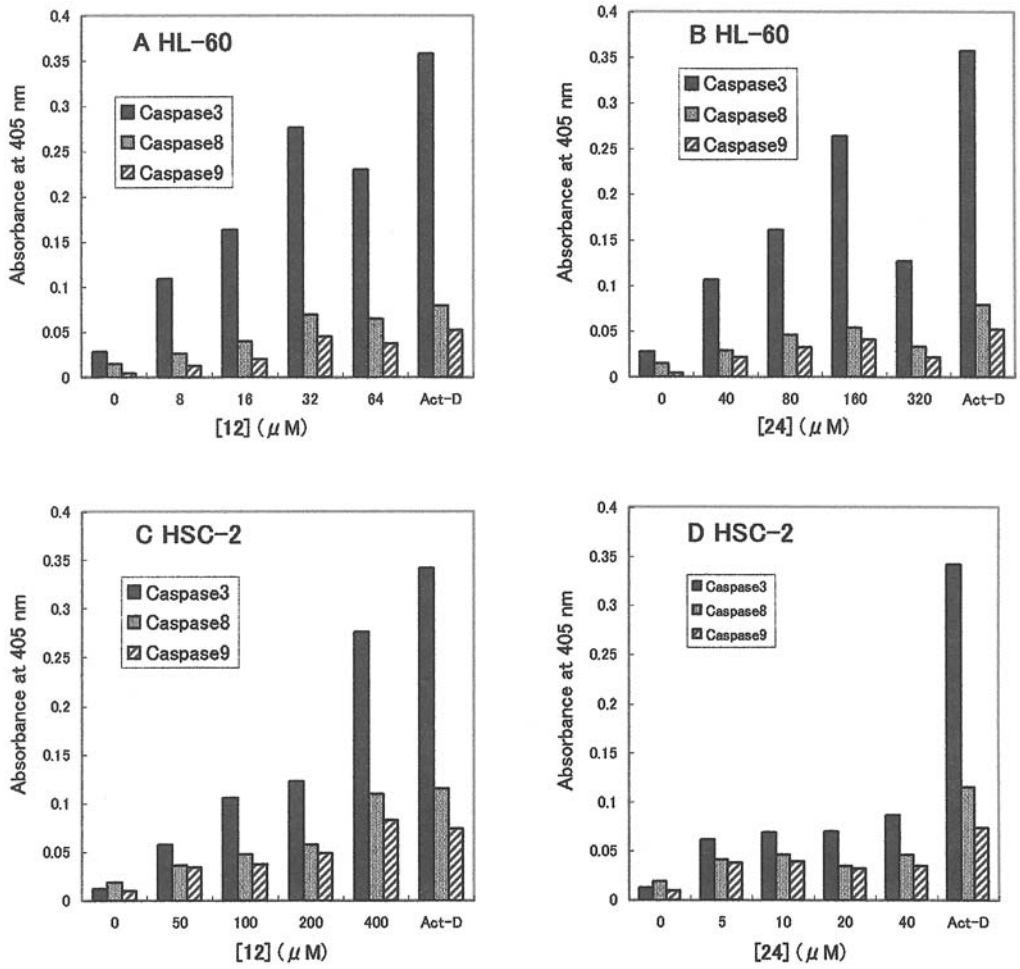

Figure 3. *Activation of caspases 3, 8 and 9 by **[12]** and **[24]**. HL-60 (A, B) and HSC-2 (C, D) cells were incubated for 4 hours without (control) or with 3-(3-guaiazulenyl)-1,5-azulenequinone **[12]** (A, C), 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone **[24]** (B, D) Act-D: 1 μg/mL actinomycin D was included in each group of figures as positive control.*

Results and Discussion

Structure and activity relationship. We investigated twenty-seven azulenequinone derivatives for their relative cytotoxicity against three human normal cells (HGF, HPC, HPLF) and four human tumor cell lines (HSG, HSC-2, HSC-3, HL-60) (Table I).

1,5-Azulenequinone **[1]**, a parent compound, showed potent (mean CC_{50} for four tumor cells (MCT= <9.1 μM) and some tumor-specific cytotoxicity (TS=>3.4) (Table I).

Halogenated derivatives of 1,5- and 1,7-azulenequinone **[2, 3, 4, 13, 14, 15, 20, 21, 22]** showed potent cytotoxicity (MCT=<7.7, <14.8, <6.4, <28.1, <24.2, <20.5, <16.7, 9.4 and 23.3 μM, respectively), but lower tumor-specific cytotoxicity (TS=>1.3, >2.1, ><2.1, >2.0, >2.1, >2.0, >1.0, >1.0 and 1.5, respectively).

In contrast to other azulenequinones, amino derivatives such as 3-amino-1,5- **[7, 8, 9, 10, 25, 26, 27]** (MCT=117.6, 93.1, >388, >192.3, 147.9, 51.7 and 26.7.4 μM, respectively) and 3-amino-1,7-azulenequinones **[17, 18, 19, 23]** (MCT=>461.1, >390.0, >248.8 and 319.2 μM, respectively) showed relatively lower cytotoxic activity.

3-Phenoxy-1,5-azulenequinone derivatives **[5, 6]** (MCT=<7.1 and <11.5 μM, respectively) showed higher cytotoxicity than the derivative **[16]** (MCT=<79.2 μM).

1,5- and 1,7-Azulenequinones generally showed higher cytotoxicity, as compared with tropolones (31) and azulene derivatives (8).

3-Phenoxy-1,5-azulenequinone **[5]**, 3-(4-nitrophenoxy)-1,5-azulenequinone **[6]**, 3-(3-guaiazulenyl)-1,5-azulenequinone **[12]**, 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone **[24]** and 7-isopropyl-3-morpholino-1,5-azulen-

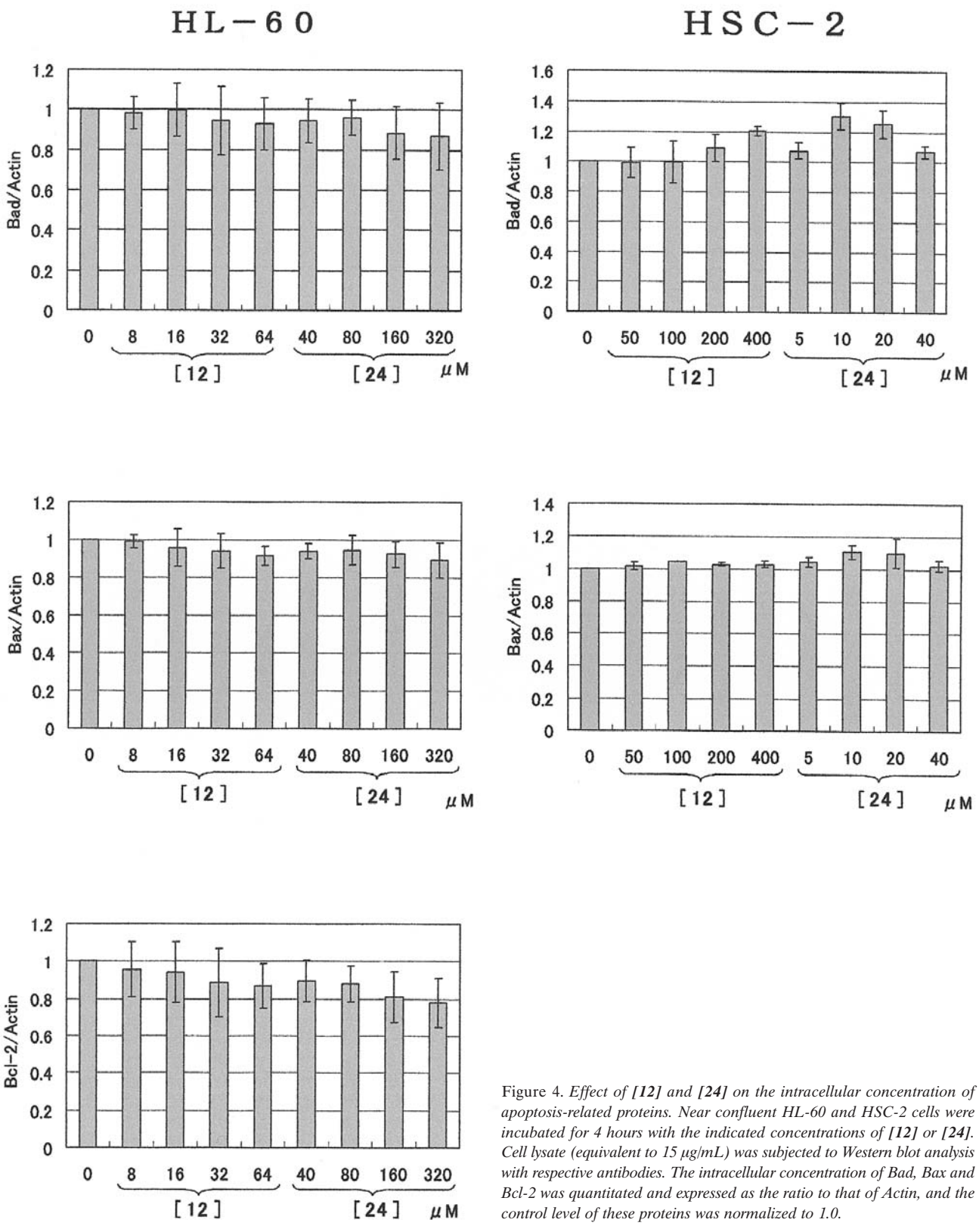

Figure 4. *Effect of **[12]** and **[24]** on the intracellular concentration of apoptosis-related proteins. Near confluent HL-60 and HSC-2 cells were incubated for 4 hours with the indicated concentrations of **[12]** or **[24]**. Cell lysate (equivalent to 15 μg/mL) was subjected to Western blot analysis with respective antibodies. The intracellular concentration of Bad, Bax and Bcl-2 was quantitated and expressed as the ratio to that of Actin, and the control level of these proteins was normalized to 1.0.*

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

	CC_{50} (μM)	EC_{50} (μM)	SI
1	368.6	>200	<1
2	56.5	>200	<1
3	164.4	>200	<1
4	13.6	>40	<1
5	7.8	>40	<1
6	4.0	>8	<1
7	103.1	>200	<1
8	64.9	>200	<1
9	479.7	>1000	<1
10	652.6	>1000	<1
11	15.6	>40	<1
12	33.7	>200	<1
13	11.7	>40	<1
14	68.6	>200	<1
15	62.2	>200	<1
16	15.5	>40	<1
17	484.2	>1000	<1
18	334.9	>1000	<1
19	294.0	>1000	<1
20	11.8	>40	<1
21	4.9	>40	<1
22	14.3	>40	<1
23	308.9	>1000	<1
24	16.5	>40	<1
25	22.7	>40	<1
26	15.1	>40	<1
27	255.7	>1000	<1
AZT	165.2	0.026	6367
ddC	3074.5	3.8	808

equinone **[26]** showed relatively higher TS value (>8.5, >4.5, 3.9, 10.2 and 5.2, respectively).

Apoptosis induction. Figure 2A shows that both **[12]** and **[24]** induced the internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, in HL-60 cells. The optimal concentrations of these compounds were 16 and 160 μM, respectively. However, neither of these compounds induced internuclesomal DNA fragmentation in HSC-2 cells (Figure 2B).

Figure 3A and B show that **[12]** and **[24]** activated caspases 3, 8 and 9 in HL-60 cells, to a level slightly lower than that attained by actinomycin D (1 μg/mL), a positive control. The optimal concentrations of these compounds were 32 and 160 μM, respectively. Figure 3C shows that **[12]** also activated caspases 3, 8 and 9 in HSC-2 cells, but required higher concentrations. Figure 3D shows that **[24]** activated these caspases, but to a much lesser extent than that attained by actinomycin D.

We next investigated the possible changes in the intracellular concentrations of pro-apoptotic proteins (Bad, Bax) and anti-apoptotic protein (Bcl-2) by Western blot analysis. Figure 4 shows that **[24]** slightly reduced the concentrations of Bcl-2 in HL-60 cells, whereas **[12]** was much less active. **[24]** elevated the concentrations of Bad and Bax more potently than **[12]** in HSC-2 cells. Bcl-2 could not be detected in HSC-2 cells with or without treatment (data not shown). The apoptosis-inducing activity of other compounds **[5, 6, 26]** will be reported elsewhere.

Anti-HIV activity. None of the twenty-seven azulenequiones showed anti-HIV activity (SI<1), in contrast to the potent anti-HIV activity of AZT (SI=6367) and ddC (SI=808), positive controls (Table II).

We found, for the first time, that among twenty-seven azulenequinone derivatives, 3-(3-guaiazulenyl)-1,5-azulenequinone **[12]** and 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone **[24]** showed both higher tumor-specific cytotoxic activity and apoptosis. These compounds activated both the mitochondria-independent extrinsic pathway (involved with caspase 8) and the mitochondria-dependent intrinsic pathway (involved with caspase 9) (32). This finding is important, since the apoptosis-inducing agents (β-diketones, α,β-unsaturated ketones, hydroxylketones) do not always show tumor-specific cytotoxicity and compounds with higher tumor-specificity do not always induce apoptosis (33-35). Further study is required to elucidate the molecular mechanism of apoptosis induction by these azulenequinones. We have recently found that azulenes and tropolones inhibited the NO production by LPS-stimulated mouse macrophage-like Raw 264.7 cells (36, 37). It remains to be investigated whether azulenequinones also inhibit the NO production by activated macrophages.

Acknowledgements

We wish to express our deep gratitude to Prof. Klaus Hafner, Technische Hochschule Darmstadt, Germany, for his very generous gift of a large amount of azulene. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No. 14370607).

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Received August 6, 2004
Accepted October 20, 2004