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Tumor-specific Cytotoxicity and Type of Cell Death Induced by Naphtho[2,3-*b*]furan-4,9-diones and Related Compounds in Human Tumor Cell Lines: Relationship to Electronic Structure

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Abstract. *A total of thirty-nine naphtho[2,3-b]furan-4,9-diones and related compounds were tested for their cytotoxicity against three human normal oral 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). 2-Acetylnaphtho[2,3-b]furan-4,9-dione **[1]** was highly cytotoxic to both normal and tumor cells, yielding low tumor-specificity. 2-Acetyl-4,9-dimethoxynaphtho[2,3-b]furan **[4]**, the 2-(3-furanoyl) benzoic acids **[5, 6]** and the 1,4-naphthoquinones **[7, 8]** showed much reduced cytototoxicity and low tumor-specificity. The introduction of phenoxy **[18]**, isopropylamino **[23]** or 2-methylpiperidino **[33]** groups to the 2-position of naphtho[2,3-b]furan-4,9-dione yielded compounds that showed the greatest tumor-specificity. These compounds, at twice or four times higher concentrations than CC_{50}, induced the activation of caspase-3, caspase-8 and caspase-9 in the HSC-2 and HL-60 cells, but not so apparently in the HSC-4 cells. However, they did not induce internucleosomal DNA fragmentation in the HSC-2 and HSC-4 cells even after 24 hours incubation and only slightly induced DNA fragmentation in the HL-60 cells. Compound **[18]** induced the production of annexin-positive cells, but did not induce microtubule-associated protein light chain 3 (LC3) accumulation in autophagosomes in LC3-green fluorescent protein (GFP)-transfected HSC-2 cells. These data suggested that naphtho[2,3-b]furan- 4,9-diones may induce the early apoptotic marker, without induction of caspase activation and DNA fragmentation in oral squamous cell carcinoma cell lines. Quantitative structure-activity relationship (QSAR) analysis suggests the applicability of the theoretical calculations such as frontier molecular orbital, dipole moments and hydrophobicity in predicting their cytotoxic activity.*

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Several naphtho[2,3-*b*]furan-4,9-diones isolated from plants have shown biological activity (1). It has been reported that 2-acetylnaphtho[2,3-*b*]furan-4,9 -dione **[1]** isolated from *Tabebuia cassinoides* (Lam.) DC (*Bignoniaceae*) was highly cytotoxic (2), and 2-methylnaphtho[2,3-*b*]furan-4,9-dione showed three times higher cytotoxicity than **[1]** (3). The activity of 2-substituted naphtho[2,3-*b*]furan-4,9-diones varies with the type of substituents on the parent naphtho[2,3-*b*]furan-4,9-dione (4). It has been reported that 2-acetyl-4,9-dimethoxynaphtho[2,3-*b*]furan **[4]**, the 2-(3-furanoyl)benzoic acids **[5, 6]** and the 1,4-naphthoquinones **[7, 8]** showed much reduced cytotoxic activity against human oral epidermal cell (KB) cells compared to the parent compound and that 2-formyl naphtho[2,3-*b*]furan-4,9-dione showed the highest cytotoxicity among forty two related compounds (4). However, the tumor specificity and type of cell death induced by naphtho[2,3-*b*]furan-4,9-diones have not yet been elucidated. Based on this background, a total of thirty nine naphtho[2,3-*b*]furan-4,9-diones and related compounds **[1, 4-10, 12-42]** (Figure 1) were investigated for their cytotoxicity 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). Since there are at least three types of cell death (apoptosis, autophagy, necrosis) (5-7), the

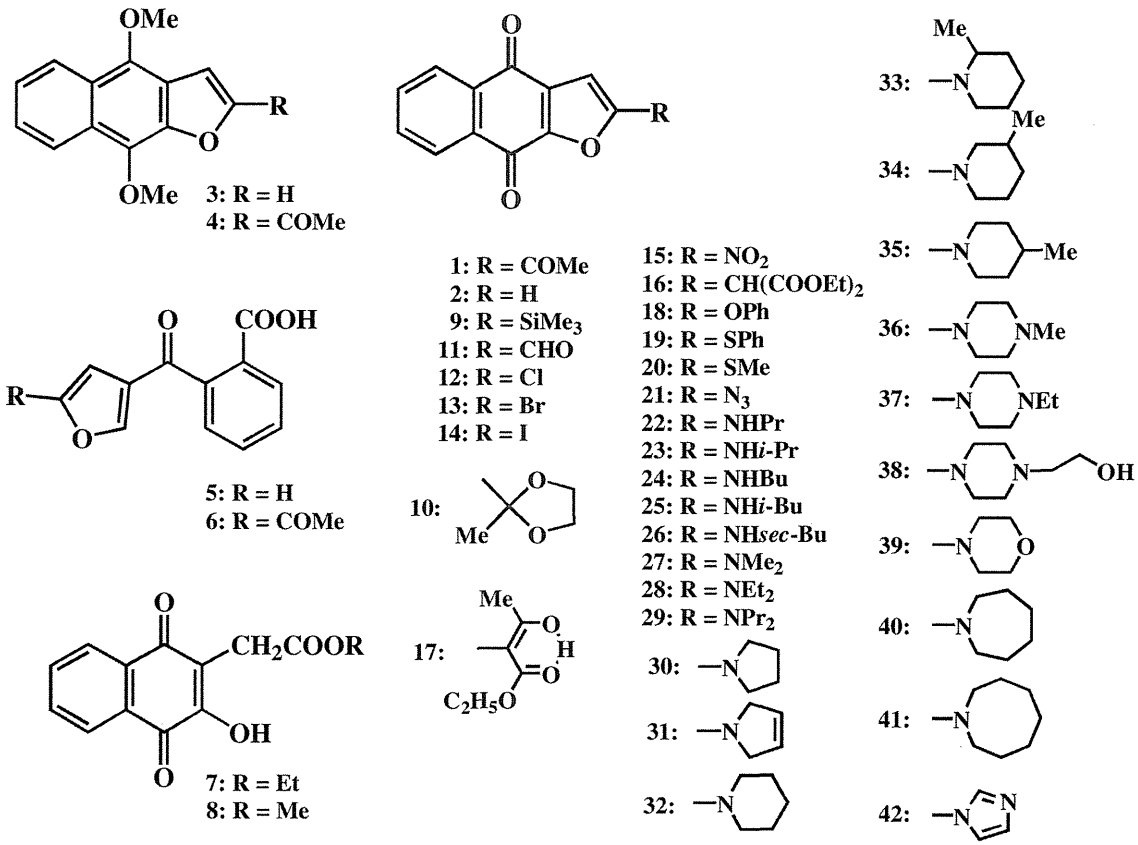

Figure 1. *Structures of the thirty-nine naphtho[2,3-b]furan-4,9-diones studied.*

type of cell death induced by these compounds was also investigated. Recently, we reported the quantitative structure-activity relationship (QSAR) between cytotoxic activity and the three parameters (hydrophobicity, $\Delta\Delta H_f$, IOH and calculated dipole moment in the gas-phase by the electrostatic potential calculations, $\mu_{ESP\text{-}G}$) of 3-benzazepine derivatives (8) and the relationship between the electronic structure and cytotoxic activity of azulenes (9), tropolones (10), azulenequinones and trihaloacetylazulenes (11). Based on our previous results, the relationship between the cytotoxic activity and the individual quantitative structure-activity relationship (QSAR) parameters was investigated. Based on a molecular orbital calculation concerning their physicochemical parameters and cytotoxic activities, the quantitative structure-activity relationship (QSAR) of the naphtho[2,3-*b*]furan-4,9-diones and related compounds was also investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the companies indicated: Dulbecco's modified Eagles 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-Aldrich Inc., St. Louis, MO, USA); annexin V (MEBCYTO-Apoptosis Kit. MBL Medical and Biological Laboratories Co., Ltd., Nagoya, Japan).

Preparation of naphtho[2,3-b]furan-4,9-diones. The naphtho [2,3-*b*]furan-4,9-diones and related compounds were synthesized according to the methods previously published (4).

Cell culture. The 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. The human promyelocytic leukemia HL-60 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. The tumor cell lines were obtained from Riken BioResource Center, Tsukuba, Ibaraki, Japan. The normal cells were prepared from periodontal tissues, according to the guideline of the Institutional Board of Meikai University Ethics Committee (No. 0707) after obtaining the informed consent from the patients. Since HGF, HPC and HPLF cells have a limited lifespan due to *in vitro* senescence (12), these cells were used for the present study at a population doubling level of 5-8.

Assay for cytotoxic activity. The cells (other than HL-60) were inoculated at 5×10^3 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 80 mM. The first well contained 800 μM test compound and was then diluted 2-fold sequentially, with 3 replicate wells for each concentration. The cells were incubated for another 24 hours and the relative viable cell number was then determined by the MTT method. In brief, the 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 the 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) (13). The A_{540} of the control cells was usually in the range from 0.40 to 0.90.

The HL-60 cells were inoculated at 7.5×10^4 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 hemocytometer under a light microscope after trypan blue staining.

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. The cells were 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*-lauroyl-sarcosinate solution) and then 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 (40 mM Tris-HCl [pH 8.0], 7.6 M NaI, 20 mM EDTA-2Na) followed by 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 [pH. 8.0], 1 mM EDTA-2Na). Each 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). A 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 (13). After staining with ethidium bromide, the DNA was visualized by UV irradiation and photographed by a charge coupled device camera (Bio Doc-It; UVP Inc., Upland, CA, USA).

Assay for caspase activation. The cells were washed with PBS(–) and lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 0.3% Noridet-P-40, 1 mM dithiothreitol). After standing for 10 minutes on ice and centrifugation for 5 minutes at 10,000 ×g, the supernatant was collected. The 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 [pNA]), 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 pNA was measured by a plate reader (13).

Assay for the appearance of early marker of apoptosis. HSC-2 cells (5×10^4/well) were plated in 8 well-chamber slides and incubated for 48 hours. The cells were then treated for 2 hours with the test samples, washed with PBS (–) and resuspended in 85 μl of binding buffer (MEBCYTO-Apoptosis Kit). Then 10 μl of Annexin V-FITC and 5 μl of propidium iodide were added. After incubation at room temperature for 15 minutes in the dark, the cells were observed by a Laser Scanning Microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany), using excitation filter 488 nm and emission filter 505-530 nm (green) and >585 nm (red).

Assay for LC3 accumulation to the autophagosome. cDNA encoding microtubule-associated protein light chain 3 (LC3) was obtained by RT-PCR from the total RNA of the HSC-2 cells with the LC3 sense primer (5' -GGGAATTCATGCCGTCGGAGAAGACCTT-3') and LC3 antisense primer (5'-GGGAATTCTAGATTACACTGACAATTT CATCC-3'). It was subcloned into the EcoRI site of pAcGFP1-C2, a GFP fusion protein expression vector (Clontech Laboratories Inc., Mountain View, CA, USA). The plasmid construct was verified by DNA sequencing using the Applied Biosystems 310 DNA sequencer (Foster City, CA, USA) (13).

HSC-2 cells were seeded at 0.5×10^6 cells/well in a 24 well plate, and the next day, the cells were transfected with a mixture of 1.5 μg of plasmid DNA and 0.7 μl of Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA, USA). After transfection for 18 hours, the cells were used for the experiment. Mock transfection was performed using the empty pAcGFP1-C2 expression vector. The GFP-LC3 transfected HSC-2 cells were observed by a Laser Scanning Microscope LSM510 (Carl Zeiss Inc.), using the excitation filter 488 nm and emission filter 505-530 nm, as described previously (13).

Theoretical calculations. The molecular orbital calculation using the parametric method 3 (PM3) was performed by application of the winMOPAC program (14). The geometries of the naphtho[2,3-*b*]furan-4,9-diones **[1-2, 9-42]** and related compounds **[3-8]** were optimized with respect to all geometrical parameters using the Broyden-Fletcher-Goldfrab-Shanno algorithm incorporated into the program. The geometries of the naphtho[2,3-*b*]furan-4,9-diones **[1-2, 9-42]** and related compounds **[3-8]** in the aqueous-solution were compared with those in the gases using the conductor-like screening model orbital (COSMO) and electrostatic potential (ESP) calculations. The COSMO procedure generates a conducting polygonal surface around the system at van der Waal's distance. The standard value of the number of the geometrical segments per atom (NSPA) was 60, and that of the dielectric constant was 78.4 at 25°C (water). The values of the dipole moment (μ_G and μ_W) in the gas-phase and in the water-solution of these compounds **[1-42]** were calculated by the ESP/PM3 and COSMO/PM3 methods. For this calculation, a DELL XPS DXG061 personal computer was used.

A partition coefficient log P was used as an index of the QSAR analysis for new drug design. A stereo hydrophobic parameter, dGW, was obtained by the PM3 method. The dGWs were defined as their free-energy changes for the association in the aqueous solution and in the gas-phase (15).

Results

Structure and activity relationship. 2-Acetylnaphtho[2,3-*b*]furan-4,9-dione **[1]** was highly cytotoxic to both normal (HGF, HPC, HPLF) and tumor cells (HSC-2, HSC-3, HSC-4, HL-60), yielding a low tumor-specificity index (TS=0.3) (Table I). 2-Acetyl-4,9-dimethoxynaphtho[2,3-*b*]furan **[4]**, the 2-(3-furanoyl)benzoic acids **[5, 6]** and the 1,4-naphthoquinones **[7, 8]** showed much reduced cytototoxicity

Table I. *Cytotoxic activity of naphtho[2,3-b]furan-4,9-diones.*

		Cytotoxic activity (CC_{50}: μM)							
		Normal human cells			Human tumor cell lines				
	MW	HGF	HPC	HPLF	HSC-2	HSC-3	HSC-4	HL-60	TS
1	240.22	0.4	0.5	1.2	0.9	0.5	5.2	2.8	0.3
4	270.28	703.3	>768.8	>753.2	71.4	645.9	548.6	>800.0	><1.4
5	216.19	>779.4	>792.2	>800.0	623.5	>760.7	>778.4	>800.0	><1.1
6	258.23	>800.0	>773.8	>800.0	>755.7	>800.0	>800.0	>800.0	><1.0
7	260.25	>800.0	679.5	704.4	551.3	543.5	606.5	494.0	>1.3
8	246.22	654.0	586.1	411.4	446.0	181.8	467.8	291.3	1.6
9	270.36	>800.0	>800.0	>853.3	>573.7	>800.0	>800.0	>425.9	><1.3
10	284.27	2.8	3.0	5.4	2.7	2.0	7.9	4.8	0.9
12	232.62	1.7	2.0	4.0	1.9	2.2	8.7	>8.0	<0.5
13	277.07	1.5	1.4	3.0	1.6	1.4	6.4	7.4	0.5
14	324.07	2.8	2.7	5.6	2.5	2.1	8.5	>8.9	<0.7
15	243.18	29.8	25.7	45.8	9.2	21.6	15.7	<6.3	>2.6
16	356.33	19.3	61.1	47.6	14.7	9.0	34.1	25.4	2.1
17	326.31	29.9	28.0	48.1	13.5	9.6	35.1	27.7	1.6
18	290.28	344.3	195.8	77.3	8.1	11.6	29.0	9.8	14.1
19	306.34	43.6	135.5	120.5	11.3	8.0	34.1	14.2	5.9
20	244.26	4.4	7.0	7.1	4.7	3.6	10.0	4.1	1.1
21	239.19	40.6	38.7	69.6	24.1	25.6	48.6	12.8	1.8
22	255.27	106.3	229.5	41.6	19.2	28.9	10.7	110.3	3.0
23	255.27	>800.0	>800.0	>853.3	23.1	29.2	25.2	11.2	>36.9
24	269.30							>800.0	
25	269.30							>800.0	
26	269.30							19.4	
27	241.25							>800.0	
28	269.30							40.4	
29	297.35							>800.0	
30	267.28	270.6	244.9	357.0	388.1	>447.8	>478.8	430.3	<0.7
31	265.27	381.6	>662.7	>800.0	516.9	>783.7	281.8	>800.0	><1.0
32	281.31	>712.2	>800.0	>738.3	77.6	559.5	194.8	19.5	>3.5
33	295.34	518.0	>549.9	464.9	53.5	84.4	32.9	14.4	>11.0
34	295.34	>800.0	>800.0	>800.0	>642.6	>776.9	>449.7	>800.0	><1.2
35	295.34	574.2	>728.6	>695.2	244.5	407.8	132.0	18.9	>3.3
36	296.33	62.5	67.4	55.4	10.6	15.9	9.0	55.6	2.7
37	310.35	58.8	77.2	57.0	8.1	15.7	8.1	33.0	4.0
38	326.35	117.9	145.5	114.0	19.3	26.8	21.8	44.1	4.5
39	283.28	>800.0	>800.0	>800.0	>607.9	>551.4	171.2	>676.1	><1.6
40	295.34	>800.0	>800.0	>800.0	179.9	>669.2	341.5	>707.2	><1.7
41	309.36	>800.0	>800.0	>800.0	558.9	>673.0	324.2	>800.0	><1.4
42	264.24	1.4	0.9	0.6	0.8	0.5	3.1	2.6	0.6

Each value represents mean of at least three independent experiments. TS: tumor specificity.

against both normal and tumor cells, with disappointingly lower tumor-specificity (TS=1.0-1.6) (Table I).

In an attempt to obtain more tumor-specific compounds, the naphtho [2,3-*b*]furan-4,9-diones were used as lead compound and several functional groups were introduced. The introduction of a trimethylsilyl group at the C-2 position markedly reduced the cytotoxicity, without elevating the tumor-specificity **[9]** (TS=1.3). Also, the introduction of 2-methyl-1,3-dioxolan-2-yl **[10]**, halogen **[12-14]**, nitro **[15]**, bulky substituent **[16, 17]** or methylthio groups **[20]** did not elevate the tumor-specificity of the parent compound (TS=0.5-2.6), nor reduce the cytotoxicity. On the other hand, the introduction of a phenoxy **[18]**, phenylthio **[19]**, isopropylamino **[23]** or 2-methylpiperidino group **[33]** enhanced the tumor-specificity to the greatest extent (TS=14.1, 5.9, >36.9 and >11.0, respectively). The introduction of azido **[21]**, propylamino **[22]**, straight **[24]** or branched **[25, 26]**, dimethylamino **[27]**, diethylamino **[28]**, diisopropylamino compounds **[26]** or those with cyclic amino residues **[30-42]** reduced the cytotoxicity to various extents without elevating the tumor-specificity

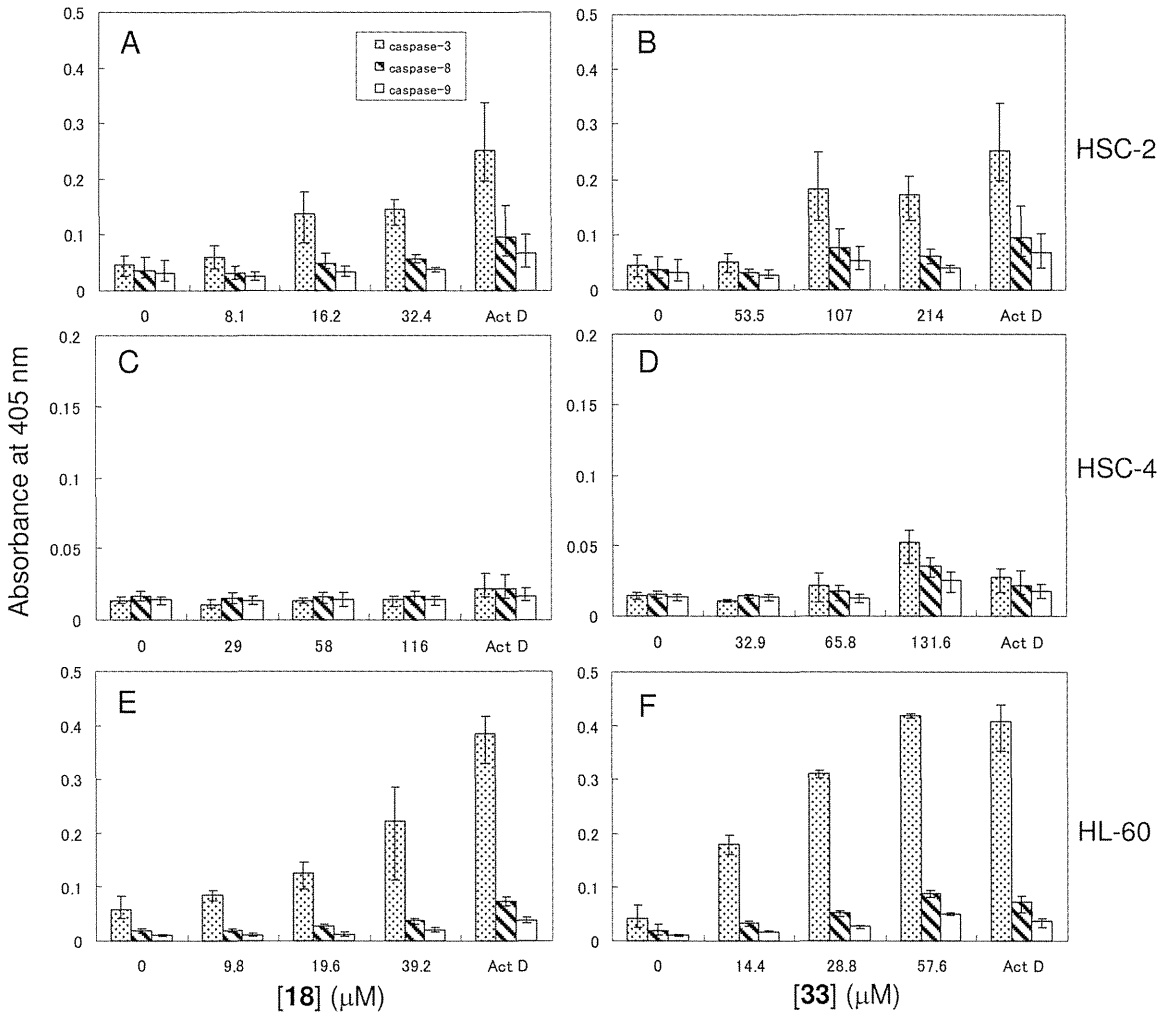

Figure 2. *Effect of two naphtho[2,3-b]furan-4,9-diones derivatives [18, 33] on caspase activation in three human tumor cell lines. HSC-2 (A, B), HSC-4 (C, D) and HL-60 cells (E, F) were incubated for 4 hours with the indicated concentrations of [18] (A, C, E) or [33] (B, D, F) or 1 μg/mL actinomycin D (ActD). Each point represents the mean±S.D. from 3-4 independent experiments.*

(TS=0.6-4.5) (Table I). It should be noted that the 2-(3-pyrrolin-1-yl) **[31]** and 2-(3-methylpiperidino) compounds **[34]** and those with a 7-member ring **[40]** and 8-member ring **[41]** were essentially inactive whereas the compound with an imidazole group **[42]** was highly cytotoxic.

Drug sensitivity of cell lines. Among the four tumor cell lines, the HL-60 cells and HSC-2 cells were generally more sensitive to the naphtho[2,3-*b*]furan-4,9-diones than the HSC-3 and HSC-4 cells (Table I).

Type of cell death induced. The type of cell death induced by the most tumor-selective compounds **[18, 23, 33]** was investigated. Compound **[18]** induced the dose-dependent activation of caspase-3, caspase-8 and caspase-9 in the HSC-2 and HL-60 cells (Figure 2A and 2E), whereas it did not activate any caspases in HSC-4 cells (Figure 2C). Similarly [33] induced the dose-dependent activation of caspase-3, caspase-8 and caspase-9 in the HSC-2 and HL-60 cells (Figure 2B and 2F), whereas it only marginally activated caspases at concentrations four times that of CC_{50} in the HSC-4 cells (Figure 2D). It should be noted that activation of caspases became prominent only at concentrations two or four times that of CC_{50} in all cases (Figure 2A, 2B, 2D, 2E, 2F).

It was unexpected that **[18]**, **[23]** and **[33]** did not induce internucleosomal DNA fragmentation in the HSC-2 (Figure 3A, 3B) and HSC-4 (Figure 3C, 3D) cells, even though the incubation time was prolonged from 6 hours to 24 hours. Compound **[18]** also failed to induce DNA fragmentation in the HL-60 cells, regardless of the incubation time (Figure 3E, 3F), while **[33]** induced internucleosomal DNA fragmentation only after 24 hours (Figure 3F).

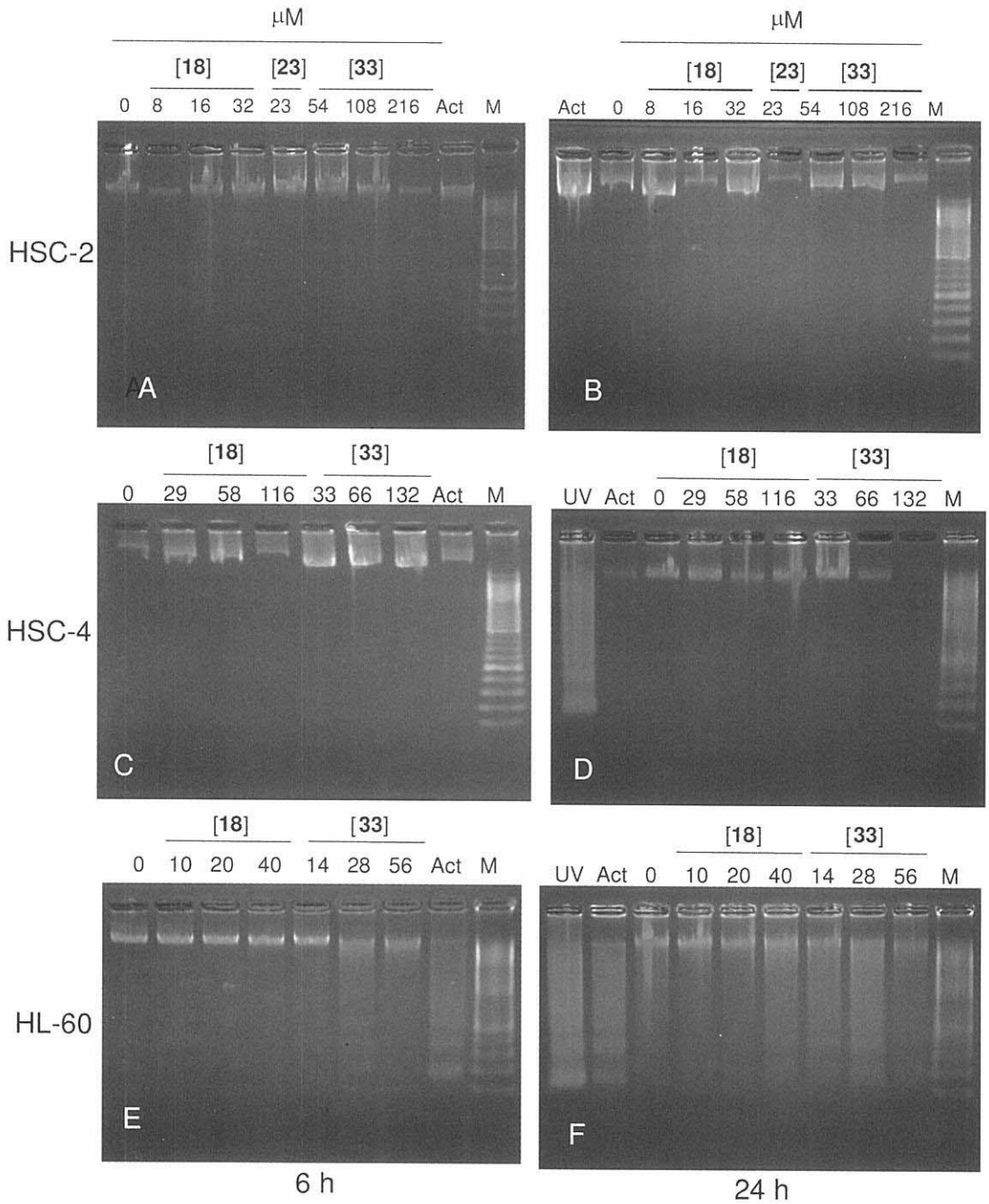

Figure 3. *Effect of three naphtho[2,3-b]furan-4,9-diones derivatives* ***[18, 23, 33]*** *on the induction of DNA fragmentation in three human tumor cell lines. Near confluent HSC-2 (A, B), HSC-4 (C, D) and HL-60 cells (E, F) were incubated for 6 (A, C, E) or 24 hours (B, D, F) with the indicated concentrations (μM) of* ***[18]****,* ***[23]*** *or* ***[33]****, or with 1 μg/mL actinomycin D (Act). DNA was then extracted and submitted to agarose gel electrophoresis. Marker DNA (indicated by M) and DNA from apoptotic HL-60 cells induced by UV irradiation (indicated by UV) were also run. Representative data from one of three independent experiments are shown.*

To determine whether **[18]** actually induced apoptosis, the appearance of phosphatidyl serine in the outer cell membrane was investigated by staining the cells with annexin and PI. Compound **[18]** was found to induce annexin-positive cells 2 hours after treatment (Figure 4A). On the other hand, **[18]** did not induce autophagosome formation, a marker for the early stage of autophagy induction (Figure 4B).

QSAR with KB cells. The cytotoxicity of forty-two naphtho[2,3-*b*]furan-4,9-diones and their related compounds against human oral epidermal (KB) cells (4) was compared with their

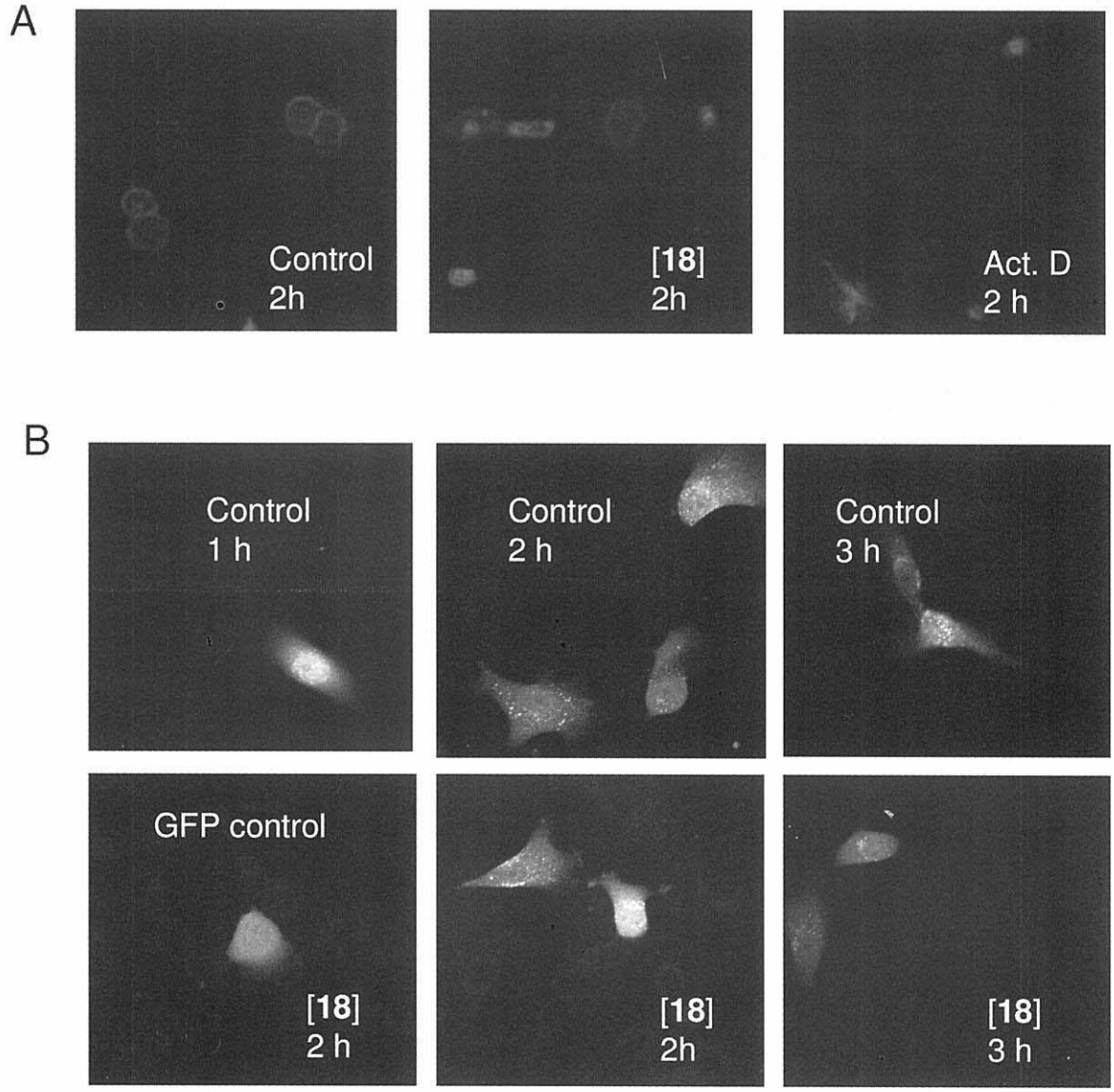

Figure 4. *Induction of apoptosis, but not autophagy in HSC-2 cells after treatment with 2-phenocynaphtho[2,3-b]furan-4,9-dione* ***[18]****. A. HSC-2 cells were treated for 2 hours without (control) or with 8.1 μM* ***[18]*** *or 1 μg/mL actinomycin D and then stained with annexin V and PI. B. HSC-2 cells transfected with empty pAcGFP1-C2 expression vector (GFP control) or with GFP-LC3 expression vector. These cells were treated for 2 or 3 hours without (control) or with 8.1 μM* ***[18]****, and then assayed for the distribution of LC3-GFP in the cytoplasm.*

electronic properties. The hydrophobicity of the whole molecule ($\Delta\Delta H_f$), the lowest unoccupied molecular orbital (LUMO) energy and the dipole moment (μ) of the naphtho[2,3-*b*]furan-4,9-diones and their related compounds **[1-42]** calculated using the PM3 method are provided in Table II. Four types of dipole moment were calculated using the PM3 method. Among the naphtho[2,3-*b*]furan-4,9-diones and their related compounds **[1-42]**, the value of $\Delta\Delta H_f$ increased in the following order: **[2]** ($\Delta\Delta H_f$=31.61 kJ/mol) < **[3]** (46.81 kJ/mol) < **[12]** (56.22 kJ/mol) < **[9]** (57.72 kJ/mol) < **[14]** (58.04 kJ/mol). < **[13]** (58.82 kJ/mol). The value of LUMO energy in the gas-phase increased in the following order: **[3]** (–0.44 eV) < **[5]** (–0.67 eV) < **[12]** (–0.69 eV) < **[6]** (–0.81 eV) < **[4]** (–0.96 eV).

The value of the dipole moment (μESP-W) in the water-solution calculated using the ESP/PM3 method also increased as follows: **[9]** (0.57 D) < **[20]** (0.85 D) < **[39]** (1.04 D) < **[33]** (1.11 D) < **[2]** (1.41 D).

The value of 50% effective dose (ED_{50}) of **[11]** against the KB cells was also the highest (ED_{50}=0.09 μg/mol), followed by **[15]** = **[42]** (ED_{50}=0.2 μg/mol), **[10]** (ED_{50}=0.4 μg/mol) and **[12]** (ED_{50}=0.5 μg/mol). Their cytotoxic activity could not be related to the individual

Table II. *QSAR parameters, observed and estimated cytotoxic activity of 4,9-dimethoxynaphtho[2,3-b]furan, 2-(3-furanoyl)benzoic acids, 1,4-naphthoquinones, and naphtho[2,3-b]furan-4,9-diones against KB cells.*

Compound No.	$\Delta\Delta H_f$	LUMO (eV)	Dipole moment	ED_{50} (μg/mol)	
	(kJ/mol)	in gas-phase	μ_{ESP-W}	obsd.	estim.
1	77.36	−1.83	5.78	1.0	2.6
2	31.61	−1.57	1.41	0.6	0.9
3	46.81	−0.44	2.47	>10	10.5
4	63.04	−0.96	2.19	6.4	6.4
5	92.57	−0.67	7.26	>10	13.3
6	113.93	−0.81	11.40	>10	15.8
7	91.41	−1.73	3.74	>10	2.0
8	93.25	−1.75	3.97	>10	2.1
9	57.72	−1.48	0.57	3.7	0.8
10	82.04	−1.58	2.11	0.4	1.7
11	80.99	−1.87	5.53	0.09	2.1
12	56.22	−0.69	2.30	0.5	0.5
13	58.82	−1.72	2.32	0.6	0.4
14	58.04	−1.71	2.48	0.7	0.5
15	92.99	−2.22	9.55	0.2	2.7
16	107.56	−1.66	4.79	3.7	3.7
17	87.76	−1.55	3.14	3.7	2.9
18	81.00	−1.57	3.05	2.2	2.5
19	68.06	−1.66	2.26	2.5	1.0
20	61.83	−1.54	0.85	0.8	0.7
21	83.58	−1.65	1.89	1.0	1.1
22	71.41	−1.42	1.67	0.6	2.5
23	71.81	−1.41	2.03	1.1	2.9
24	73.20	−1.42	1.71	0.7	2.5
25	70.10	−1.40	1.95	0.8	2.8
26	70.89	−1.39	2.55	1.6	3.4
27	68.90	−1.39	1.71	1.0	2.7
28	67.88	−1.35	1.97	3.9	3.2
29	67.08	−1.30	3.13	>10	4.5
30	69.94	−1.37	1.84	1.4	3.0
31	73.33	−1.40	1.60	>10	2.5
32	68.75	−1.35	2.10	1.8	3.3
33	66.04	−1.46	1.11	1.7	1.6
34	67.58	−1.37	1.93	>10	3.2
35	59.21	−1.36	2.31	4.3	3.2
36	84.46	−1.40	1.68	1.1	2.9
37	82.22	−1.40	1.85	1.6	3.0
38	104.76	−1.44	2.65	0.6	3.8
39	86.00	−1.47	1.04	0.7	1.8
40	67.92	−1.34	2.11	6.7	3.4
41	67.74	−1.33	2.07	>10	3.4
42	88.39	−1.80	5.79	0.2	3.0

$\Delta\Delta H_f$=hydrophobicity of whole molecule. Dipole moment in Debye units. obs., observed; estim., estimated from the corresponding equation. ED_{50}: 50% effective dose.

Table III. *QSAR parameters, observed and estimated cytotoxic activity of naphtho[2,3-b]furan-4,9-diones against HSC-2 cells.*

Compound No.	$\Delta\Delta H_f$	LUMO (eV)	Dipole moment	CC_{50}:μM: HSC-2	
	(kJ/mol)	in gas-phase	μ_{ESP-W}	obsd.	estim.
1	77.36	−1.83	5.78	0.9	16.2
4	63.04	−0.96	2.19	71.4	60.3
10	82.04	−1.58	2.11	2.7	5.6
12	56.22	−0.69	2.30	1.9	2.8
13	58.82	−1.72	2.32	1.6	1.8
14	58.04	−1.71	2.48	2.5	1.3
15	92.99	−2.22	9.55	9.2	9.1
16	107.56	−1.66	4.79	14.7	14.8
17	87.76	−1.55	3.14	13.5	12.4
18	81.00	−1.57	3.05	8.1	14.8
19	68.06	−1.66	2.26	11.3	7.8
20	61.83	−1.54	0.85	4.7	4.4
21	83.58	−1.65	1.89	24.1	−5.8
22	71.41	−1.42	1.67	19.2	19.7
23	71.78	−1.41	2.03	23.1	23.1
32	68.75	−1.35	2.10	77.6	29.8
33	66.04	−1.46	1.11	53.5	7.3
36	84.46	−1.40	1.68	10.6	19.8
37	82.22	−1.40	1.85	8.1	23.4
38	104.76	−1.44	2.65	19.3	15.9
42	88.39	−1.80	5.79	0.8	20.5

$\Delta\Delta H_f$=hydrophobicity of whole molecule. Dipole moment in Debye units. obs., observed; estim., estimated from the corresponding equation.

QSAR parameters, such as $\Delta\Delta H_f$, LUMO energy or μ_{ESP-W}. The correlation coefficient (r^2) and the Fisher statistic (F) are important in assessing the "goodness" of a regression fit. In order to obtain more quantitative characteristics of the "goodness" of a model, the well-known Fisher statistic value was used. Of the forty-two naphtho[2,3-*b*]furan-4,9-diones and their related compounds, 2-formylnaphtho[2,3-*b*]furan-4,9-dione **[11]** showed the highest cytotoxicity against the KB cells. In order to obtain a quantitative correlation between the cytotoxic activity and the electronic properties, the coefficient of the multiple determinations and the F value were calculated. The structure-activity relationship analysis revealed that the hydrophobicity of the molecule ($\Delta\Delta H_f$), the LUMO energy ($E_{L(G)}$) in the gas-phase and the dipole moment (μ_{ESP-W}) in aqueous-solution might significantly contribute to cytotoxic activity. Consequently, the following correlation (Equation **1**) was obtained for the cytotoxicity against KB cells.

$$ED_{50}=11.19+0.019 \times \Delta\Delta H_f + 8.025 \times E_{L(G)} + 0.794 \times \mu_{ESP-W} \quad \text{(Equation 1)}$$

n=5 (**4, 12, 16, 21, 33**), r^2=0.999, F=1178.2 > F (3, 1; 0.05)=215.71.

QASR with HSC-2 cells. The $\Delta\Delta H_f$, LUMO energy and μ of the naphtho[2,3-*b*]furan-4,9-diones and their related compounds are summarized in Table III. Of the twenty-one naphtho[2,3-*b*]furan-4,9-diones, 2-acetylnaphtho[2,3-*b*]furan-4,9-dione **[1]** and 2-(1-imidazolyl) compound **[42]** were the

most cytotoxic against the HSC-2 tumor cell line (CC_{50}=0.9 and 0.8 μM, respectively). The correlation coefficient (r2) and F value between the CC_{50} values against the HSC-2 cells for the twenty-one naphtho[2,3-*b*]furan-4,9-diones and related compounds **[1, 4, 10, 12-23, 32-33, 36-38, 42]**, using the three electronic parameters of $\Delta\Delta H_f$, lowest unoccupied molecular orbital (LOMO) energy ($E_{L(G)}$) and μ_w in aqueous-solution, were calculated to be 0.999 and 701.57 (> F(3, 26; 0.05) = 215.71), respectively. Since the correlation coefficient (r^2) and F-values of these compounds was the highest, this model was accepted. In the case of the twenty-one naphtho[2,3-*b*]furan-4,9-diones and related compounds, the following correlation equations **[2]** were obtained for the HSC-2 cell lines:

$$CC_{50}=+145.44 + 0.11 \times \Delta\Delta H_f + 96.54 \times E_{L(G)} + 9.41 \times \mu_{ESP\text{-}G} \quad \text{(Equation 2)}$$

n=5 (**13, 15, 16, 20, 23**), r^2=0.999, F=701.57.

The CC_{50} values estimated from the corresponding equations are shown in Table III. The expected CC_{50} values of most of the azulenequinones against the HSC-2 cells generally matched those for the CC_{50} values for the corresponding compounds except **[4, 21, 32]**. Depending on the type of cells, different compounds among one series of derivatives strayed from the regression lines.

Discussion

Naphtho[2,3-*b*]furan-4,9-dione showed much higher cytotoxic activity to both human normal and tumor cells than 2-acetyl-4,9-dimethoxynaphtho[2,3-*b*]furan **[4]**, 2-(3-furanoyl)benzoic acids **[5, 6]** and 1,4-naphthoquinones **[7, 8]** and the introduction of 3-pyrrolin-1-yl **[31]**, 2-(3-methylpiperidino) **[34]**, a 7-member ring **[40]** or an 8-member ring **[41]** significantly reduced the cytotoxicity of naphtho[2,3-*b*] furan-4,9-dione, further confirming the previous report (4). The naphtho [2,3-*b*]furan-4,9-diones structure was shown to be essential for exerting the highest cytotoxicity. The present study demonstrated for the first time that a phenoxy **[18]**, isopropylamino **[23]** or 2-methylpiperidino group **[33]** in the C-2 position of naphtho[2,3-*b*]furan-4,9-dione enhanced the tumor-specificity to the greatest extent. These tumor-specific compounds induced only early apoptotic markers such as the appearance of phosphatidyl serine in the outer cell membrane (as measured by annexin-positivity) without the induction of rather later apoptotic markers (such as caspase activation and internuclesomal DNA fragmentation) or autophagic markers (such as autophagosome formation detected by LC3 accumulation). The lower level of apoptosis induction by these compounds may have been due to the presence of the 1,4-naphthoquinones. We have previously reported that vitamin K_2 derivatives with 1,4-naphthoquinone structure (MK-2) induced little or no apoptotic markers in HL-60 and HSC-2 cells, as compared with geranylgeraniol, the isoprenyl unit present in the vitamin K_2 series (16). This further supported the suggestion that the presence of α,β-unsaturated ketone triggers non-apoptotic cell death (7).

The theoretical calculations such as frontier molecular orbital, dipole moments and $\Delta\Delta H_f$ may be applicable in predicting the cytotoxic activity of. naphtho[2,3-*b*]furan-4,9-diones and related compounds

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