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Cytotoxic Activity of Styrylchromones against Human Tumor Cell Lines

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Abstract. *A total of 6 newly-synthesized styrylchromones (SC-1 ~ SC-6) were compared for their cytotoxic activity against three normal oral human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) and four human tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukemia HL-60). All compounds showed higher cytotoxic activity against tumor cell lines than against normal cells. Among the 6 compounds, SC-3, SC-4 and SC-5, which have one to three methoxy groups, showed higher tumor specificity and water solubility. The cytotoxic activity of SC-3 and SC-5 was slightly reduced by a lower concentration of NADH, a quinone reductase, but that of SC-3 was enhanced by higher concentrations of NADH, possibly due to demethylation of the methoxy groups. Agarose gel electrophoresis demonstrated that SC-3 and SC-5 induced internucleosomal DNA fragmentation in HL-60 cells and production of large DNA fragment in HSC-2 cells. Both SC-3 and SC-5 enhanced the enzymatic activity to cleave the substrates for caspases 3, 8 and 9, suggesting the activation of both extrinsic and intrinsic apoptosis pathways. ESR spectroscopy showed that these compounds produced no detectable amount of radical and did not scavenge superoxide anion generated by the hypoxanthine-xanthine oxidase reaction. The highly tumor-specific cytotoxic action and apoptosis-inducing capability of SC-3 and SC-5 suggest their applicability for cancer chemotherapy.*

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We have screened a variety of natural and synthetic materials which can effectively and selectively kill human oral tumor cell lines. Among natural substances, lignins showed very low cytotoxic activity, but synergistically stimulated the cytotoxic action of vitamins C (1) and K (2). Macrocyclic hydrolysable tannins (3) and isoprenylated flavones (4, 5) effectively induced apoptotic cell death (DNA fragmentation, caspase activation) in oral tumor cell lines.

Synthetic compounds, such as benzothiepins, benzoxepins, 5-benzoylimidazoles (6-8) and 2-amino-methylene-3(2*H*)-benzofuranones (9) showed higher cytotoxic activity against human oral tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG) than against human normal cells (gingival fibroblast HGF), suggesting the tumor-specific cytotoxic action of these compounds. Among 2-styrylchromone derivatives, hormothamnione showed cytotoxicity toward P388 mouse leukemic and HL-60 human promyelocytic leukemic cell lines *via* inhibition of RNA synthesis (10). Oral administration of several compounds, all substituted at C-6 with a carboxylic acid group, showed anti-allergic activity (11). Synthetic 2-styrylchromone derivatives, with carboxylate substitution at C-6, and styryl-chromone-8-acetic acid derivatives showed anticancer activity (12). These data suggest the possible application of styrylchromone derivatives as anticancer drugs. We newly synthesized a total of 6 styrylchromones (Figure 1) (13, 14). We investigated here whether they display tumor-specific cytotoxic action, using three normal oral human cells (HGF, pulp cell HPC, human periodontal ligament fibroblast HPLF) and four human tumor cell lines (HSC-2, HSC-3, HSG, HL-60) and, if so, whether they can induce apoptosis-associated characteristics such as internucleosomal DNA fragmentation and caspase activation in the tumor cell lines. We also investigated whether radical-mediated reactions are involved in the induction of cytotoxicity, using ESR spectroscopy.

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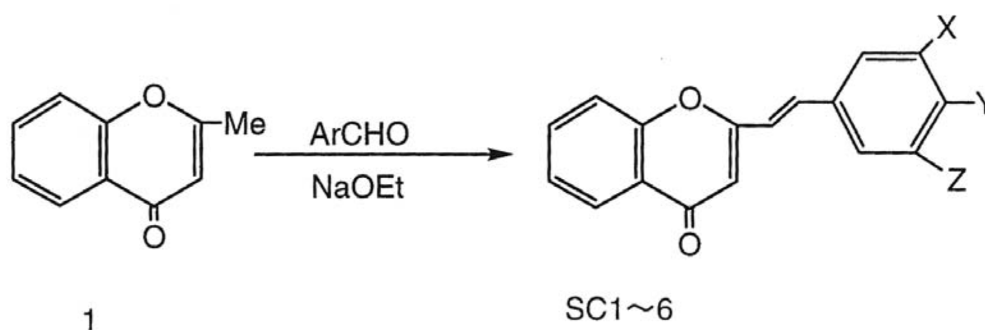

Compound	X	Y	Z	Yield(%)	mp(℃)	PMR($CDCl_3$) δ(ppm)(Ethylene proton)	Formular & molecular weight
SC-1	H	H	H	80	141-143[13)]	6.79(1H,d,J=16.1Hz)	$C_{17}H_{12}O_2$=248.27
SC-2	H	Me	H	57	164[13)]	6.73(1H,d,J=16.1Hz)	$C_{18}H_{14}O_2$=262.29
SC-3	H	OMe	H	80	139-140[13)]	6.64(1H,d,J=15.9Hz)	$C_{18}H_{14}O_3$=278.29
SC-4	H	OMe	OMe	78	163-164[13)]	6.65(1H,d,J=15.9Hz)	$C_{19}H_{16}O_4$=308.32
SC-5	OMe	OMe	OMe	85	210-211	6.70(1H,d,J=15.9Hz)	$C_{20}H_{18}O_5$=338.34
SC-6	O-CH_2-O		H	80	209-210	6.62(1H,d,J=16.2Hz)	$C_{18}H_{12}O_4$=292.28

Figure 1. *Structures of styrylchromones.*

Materials and Methods

Materials. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci, Lenexa, KS, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem, Ind, Ltd, Osaka, Japan); 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD) (Sigma Chem. Ind., St. Louis, MO, USA); RNase A, proteinase K (Boehringer Mannheim, Germany); DEVD-*p*NA (*p*-nitroanilide), IETD-*p*NA, LEHD-*p*NA (MBL, Nagoya, Japan); diethylenetriaminepentaacetic acid (DETAPAC) (Wako Pure Chem, Ind, Ltd); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan).

General procedure for the synthesis of 2-styrylchromones (SC-1 ~ 6). To a solution of Na (0.46 g, 0.02 mol) in 10 mL of EtOH was added 1.6 g of 2-methylchromone (0.01 mol) and 0.012 mol of aromatic aldehyde. The solution was left to stand at room temperature for 12 hours, then poured into ice-water and filtered to obtain the precipitate, which was washed with water and recrystallized from MeOH to give 2-styrylchromones (SC-1 ~ 6) (Figure 1).

Cell culture. All adherent cells [HSC-2, HSC-3, HSG (purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank), HGF, HPC, HPLF] were cultured in DMEM medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Normal cells (HGF, HPC, HPLF) were prepared from the periodontal tissue, according to the guideline of the Meikai University Ethics Committee (No. 0206). Since normal human cells have a limited *in vitro* life-span (15), these cells were used in the present study at 6-7 population doubling level (PDL). HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

Assay for cytotoxic activity. Near confluent cells grown in 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson) were incubated for 24 hours with various concentrations of samples. The cells were washed once with phosphate-buffered saline (PBS), and incubated for a further 4 hours with fresh culture medium containing 0.2 mg/mL MTT. After removing the medium, the cells were lysed with 100 μL DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using microplate reader with a Star/DOT Matrix Printer JL-10 (Labsystem Multiskan®, Biochromatic, Helsinki, Finland). Viable cell number of HL-60 cells was determined by trypan blue exclusion. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Table I. *Cytotoxic activity of styrylchromones.*

	CC_{50} (μM)							
	Tumor cell lines				Normal cells			
	HSC-2	HSC-3	HSG	HL-60	HGF	HPC	HPLF	SI [a)]
(Exp. I)								
SC-1	460	198	315	129	669	1097	504	2.8
SC-2	263	233	1294	115	1431	2767	527	3.3
SC-3	133	43	101	50	1665	899	835	13.8
SC-4	393	97	393	62	750	2721	2318	8.2
SC-5	92	41	30	163	2207	2243	2219	27.3
SC-6	599	>3425	1784	>3425	1198	2377	2086	<0.8
(Exp. II)								
SC-1	367	476	427		706	819	657	1.7
SC-2	214	649	847		>3817	1702	2057	>4.4
SC-3	317	191	86		1198	2331	2421	10.0
SC-4	2244	282	331		295	>3247	2588	>2.1
SC-5	59	278	139		1787	317	1479	7.5
SC-6	216	3253	2620		>3425	3051	1904	1.4

a) Selectivity index (SI) of tumor specificity was defined by the following equation.

(Exp I.)

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

(Exp. II)

$$SI = \frac{CC_{50}\,(HGF) + CC_{50}\,(HPC) + CC_{50}\,(HPLF)}{CC_{50}\,(HSC\text{-}2) + CC_{50}\,(HSC\text{-}3) + CC_{50}\,(HSG)}$$

Detection of morphological changes. Cells were prepared on slides by use of Shandon Cytospin II (Baltimore, Maryland, USA), and they were stained by May-Gruenwald's solution and Giemsa solution (30 min) (16).

Assay for DNA fragmentation. The cells were pelleted, lysed and digested with RNase A and proteinase K. DNA was isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis. The DNA isolated from the UV-irradiated HL-60 cells was run in parallel, as a marker of oligonucleosomal DNA fragments (17).

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

Assay for radical intensity. The radical intensity of the test sample in 0.1 M $NaCO_3/NaHCO_3$ buffer (pH 10.0) or in 0.1 M KOH (pH 12.5) was determined at 25˚C, using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency). Instrument settings; center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500 or 630, time constant, 0.03 or 0.1 second; scanning time, 2 or 4 minutes .

For determination of superoxide anion radical (O_2^-) produced by hypoxanthine (HX) and xanthine oxidase (XOD) reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 0.5 mM DETAPAC 20 μL, 50% DMPO 10 μL, 0.1 M PB 20 μL, sample (in DMSO) 50 μL, XOD (0.5 U/ml in PB) 50 μL], the gain, time constant and scanning time were changed to 500, 0.1 second and 2 minutes, respectively. The radical intensity was determined by ESR spectroscopy 1 minute after mixing (18).

Computational details. Theoretical calculations were carried out, using the restricted Hartree-Fock level (HPL) PM3 semi-empirical method, as implemented in the MOPAC program on a Tektronix CAChe work system (version 3.8). Log P was calculated, using a relative permittivity of 78.4 for water for the COSMO model. Giometries were optimized in internal coordinates and were terminated when the Herberts test was satisfied in the eigenvector following method (EP) (19, 20).

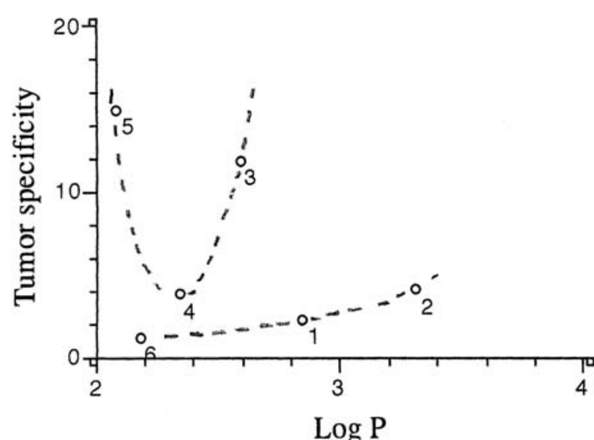

Figure 2. *Relationship between tumor specificity and log P.*

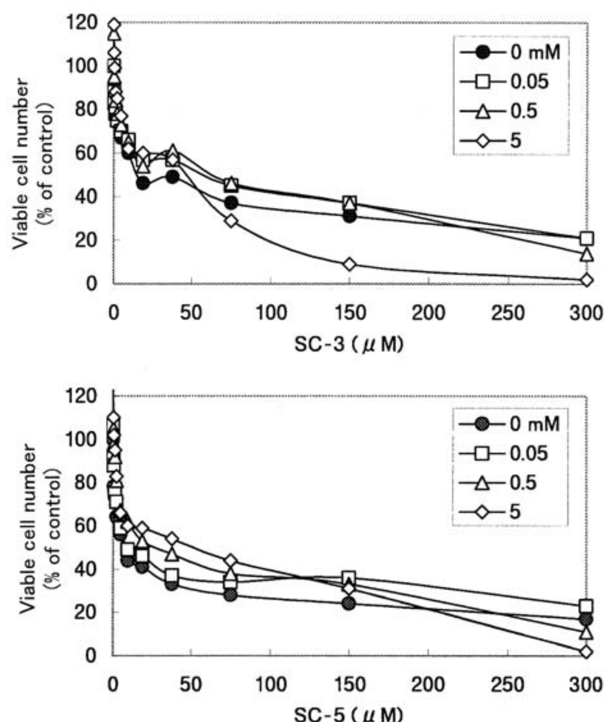

Figure 3. *Effect of NADH on the cytotoxic activity of SC-3 and SC-5. Near confluent HSC-2 cells were incubated for 24 hours with the indicated concentrations of SC-3 or SC-5 in the presence of 0 (●), 0.05 (□), 0.5 (△) or 5 mM (◇) NADH, and the relative viable cell number was then determined by MTT method. Control A_{540} value was 0.998 (for SC-3) and 0.873 (for SC-5), respectively.*

Results

Tumor-specific action. All six compounds (SC-1 ~ SC-6) showed higher cytotoxicity against tumor cell lines as compared with normal cells (Table I). The tumor-specific cytotoxicity of these compounds was evaluated by selectivity index (SI). SC-3, SC-4 and SC-5, particularly SC-3 and SC-5, showed much higher tumor-specific cytotoxic activity [SC-3 (SI=10.0-13.8), SC-4 (SI=2.1-8.2), SC-5 (7.5-27.3)] than the other compounds [SC-1 (SI=1.7-2.8), SC-2 (SI=3.3-4.4), SC-6 (SI=0.8-1.4)]. The compounds with methoxy groups (SC-3, 4, 5) showed greater tumor-specific cytotoxicity, despite higher water solubility (lower log P value) (Figure 2).

The cytotoxic activity of SC-3 and SC-5 was slightly reduced by a lower concentration of NADH (0.05-0.5 mM), but that of SC-3 was enhanced by higher concentrations of NADH (5 mM) (Figure 3).

Induction of apoptosis. Agarose gel electrophoresis showed that SC-3 and SC-5 induced internucleosomal DNA fragmentation (Figure 4A) and production of apoptotic bodies (data not shown) in HL-60 cells. The DNA fragmentation was more pronounced at 24 hours than at 6 hours (Figure 4A). On the other hand, SC-3 and SC-5 induced the production of large DNA fragments without induction of internucleosomal DNA fragmentation in HSC-2 cells (arrows in Figure 4B).

We next investigated whether these compounds activate three representative caspases (caspases 3, 8, 9), monitoring the ability of cell lysate to cleave the substrates for them. Figure 5 shows that both SC-3 and SC-5 actually activated caspases 3, 8 and 9. The activation of caspases by SC-3 exceeded that achieved by UV irradiation (positive control) (17).

Radical production. ESR spectroscopy showed that all six styrylchromones (SC1-6) produced no detectable amount of radical under alkaline condition (pH 10, 12.5) (Figure 6A) and did not scavenge superoxide anion generated by the hypoxanthine-xanthine oxidase reaction (Figure 6B).

Discussion

The present study demonstrates that all 6 newly-synthesized styrylchromones (SC-1 ~ SC-6) showed various extents of tumor-specific cytotoxicity. Among these compounds, SC-3 and SC-5 showed the highest tumor specificity, followed by SC-4. Since these compounds have one to three methoxy group(s), these methoxy groups may be involved in the induction of tumor-specific cytotoxicity. 2-Styrylchromones have recently been identified as antirhinovirus flavonoids. The introduction of a hydroxy or methoxy group in position 3 of the chromone ring enhances the cytotoxicity and antiviral activity (21). Conversely, the cytotoxic activity of flavonoids with a styrylchromone structure against tumor cells is not produced by the lack of a methoxy group (22). In sytylchromone-related compounds, the methoxy groups

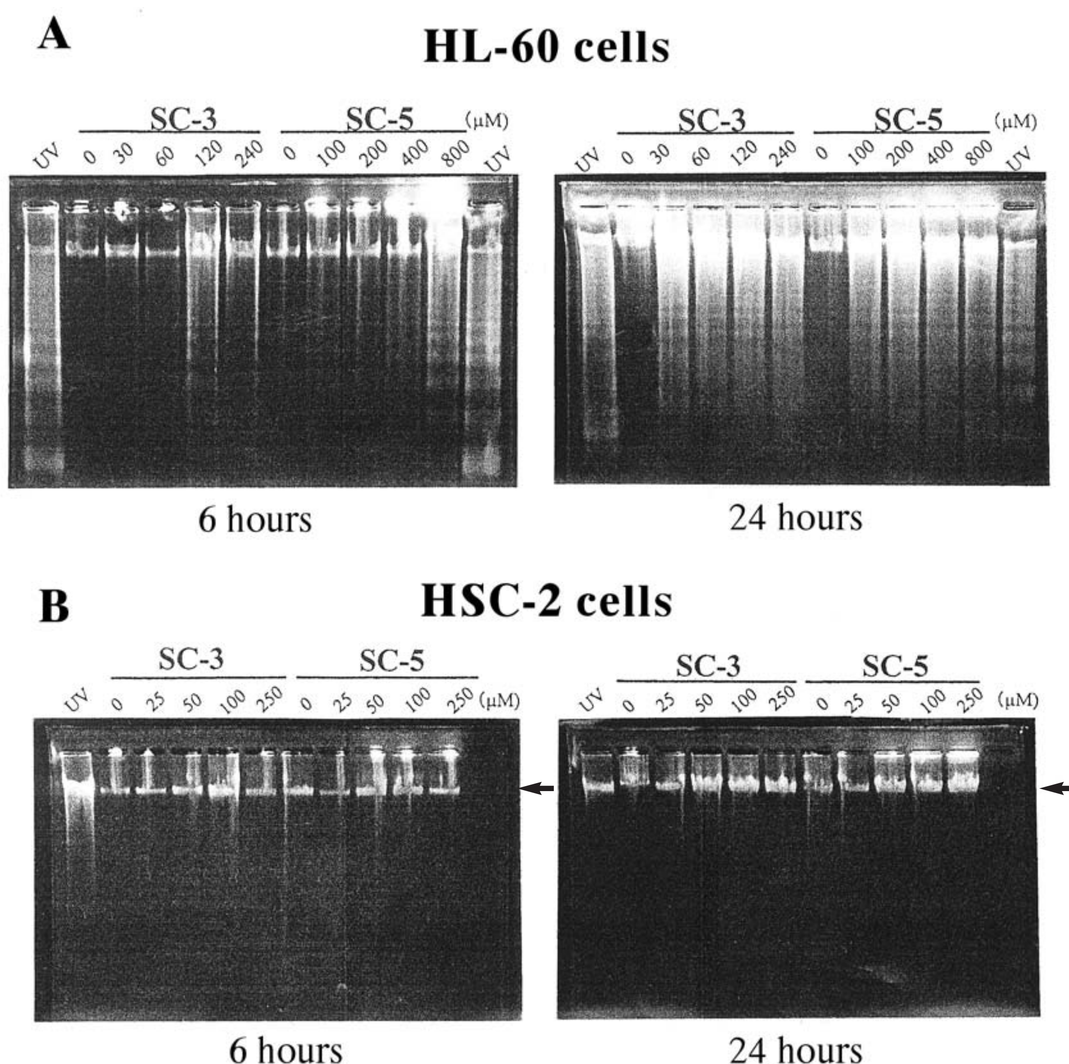

Figure 4. *Induction of internucleosomal DNA fragmentation by SC-3 and SC-5. Near confluent HL-60 (A) and HSC-2 (B) cells were incubated for 6 or 24 hours with the indicated concentrations of SC-3 or SC-5. DNA was then extracted and applied to agarose gel electrophoresis.*

may be essential for the induction of cytotoxicity and apoptosis. The enzymatic mechanism involving oxidation to the ortho-quinone has been previously reported in methoxyphenols where the methoxy group is demethylated (23). We found that the cytotoxicity of SC-3 was significantly enhanced by the addition of a higher concentration (5 mM) of NADH, but reduced by lower concentrations of NADH. The cytotoxicity and apoptosis-inducing activity of butylated hydroxytoluene (BHT) was significantly enhanced by NADH, possibly due to the reduction of BHT intermediates to cytotoxic semiquinone in the cells (24). The present study strongly suggests that the addition of NADH stimulates the formation of orthoquinone in styrylchromones (SC-4, SC-5) and quinone in SC-3 by demethylating the methoxy groups enzymatically,

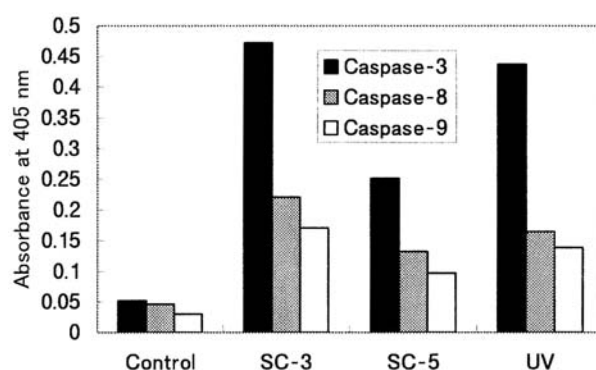

Figure 5. *Activation of caspases by SC-3 and SC-5 in HL-60 cells. HL-60 cells were incubated for 4 hours with 100 μg/mL of SC-3 or SC-5 and caspase activity was determined by cleavage of respective substrates. UV, positive control of apoptotic cells induced by UV irradiation (6J/m²/min, 1 min) (17).*

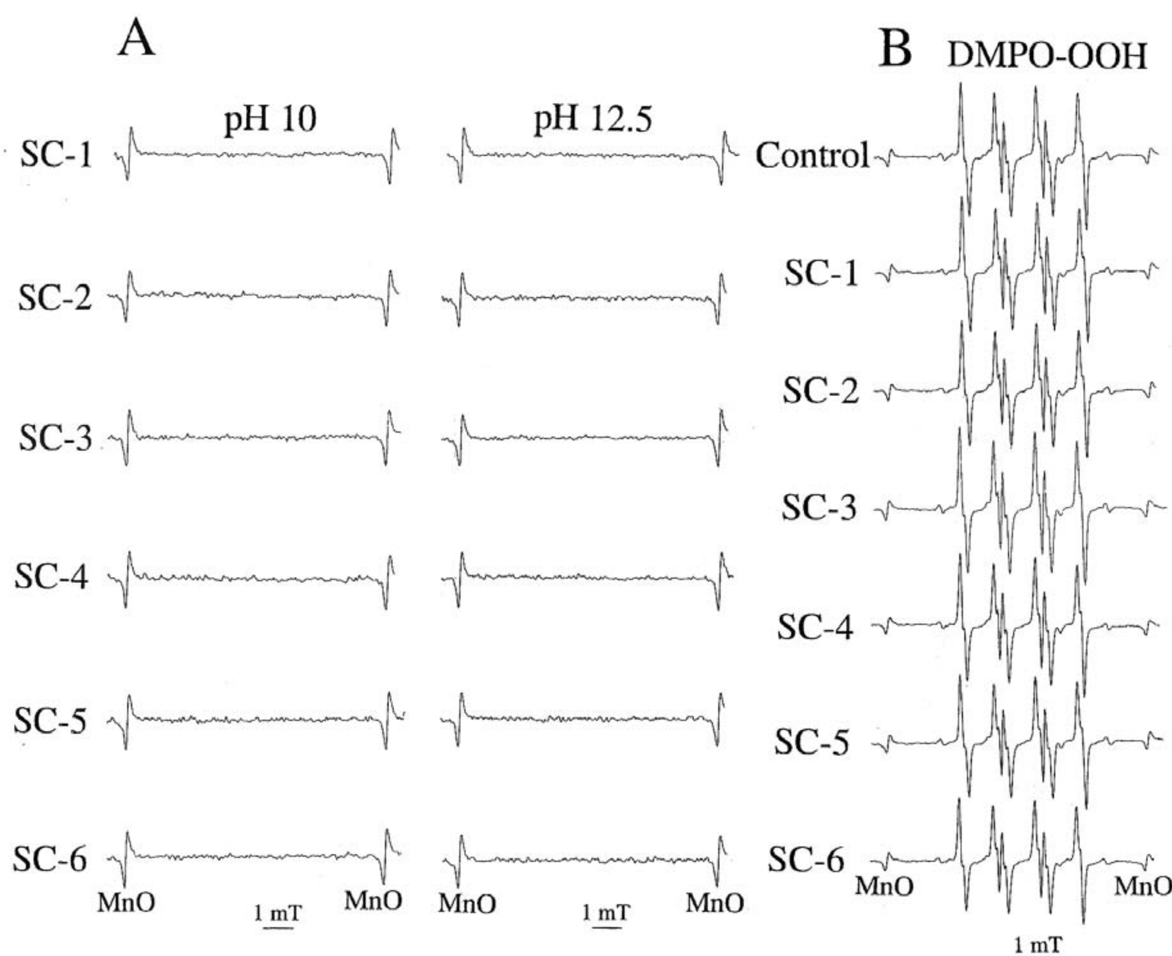

Figure 6. *Radical intensity and O_2^- scavenging activity of styrylchromones. (A) Sample (final: 2 mg/mL) was added. (B) Sample (final: 2 mg/mL) was added.*

even though styrylchromones were not oxidized under alkaline conditions.

SC-3 and SC-5 induced apoptosis characterized by internucleosomal DNA fragmentation and caspase 3 activation. Since these compounds activated both the non-mitochondrial extrinsic pathway (involved with caspase 8) and the mitochondrial intrinsic pathway (involved with caspase 9) (25), they may induce the activation of caspase 3 by both of these pathways and finally activate the DNase, which induces internucleosomal DNA fragmentation (26, 27). Our recent study demonstrated that apoptosis-inducing agents do not always show tumor-specific cytotoxicity and that compounds with higher tumor-specific cytotoxicity do not always induce apoptosis (manuscript in preparation). In this sense, SC-3 and SC-5, which show both tumor-specificity and apoptosis-inducing activity, are very promising for future application to cancer research. This may be related to the formation of cytotoxic orthoquinone due to enzymatical demethylation of methoxy groups. In contrast, SC-6 did not show any tumor-specificity, suggesting that substituents of OCH_2O were not dehydrolized due to ether bonding and, consequently, did not form cytotoxic orthoquinone. We found that fibroblasts from oral origin were less sensitive to styrylchromones. Fibroblastoid cells exhibit high resistance to quinone compounds because of the high activity of quinone reductase (28). The clinical value of compounds with lower tumor-specificity may be low, even though they can induce apoptosis in certain types of tumor cell lines.

SC-3 and SC-5 induced internucleosomal DNA fragmentation only in HL-60 cells, but not in HSC-2 cells, although these cell lines are relatively sensitive to many apoptosis-inducing agents. This suggests that induction of either internucleosomal DNA fragmentation or production of large DNA fragments may depend on the cell types rather than drug sensitivity.

We suggest that SC3 and SC5 should further be tested as possible candidates for cancer chemotherapy.

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