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Cytotoxic Activity of Deferiprone, Maltol and Related Hydroxyketones against Human Tumor Cell Lines

EIJI YASUMOTO[1], KENSUKE NAKANO[1], TOHRU NAKAYACHI[1], SUFI REZA MD. MORSHED[1], KEN HASHIMOTO[1], HIROTAKA KIKUCHI[2], HIROFUMI NISHIKAWA[2], MASAMI KAWASE[3] and HIROSHI SAKAGAMI[1]

[1]*Department of Dental Pharmacology and*
[2]*Department of Endodontics, Meikai University School of Dentistry, Sakado, Saitama;*
[3]*Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan*

Abstract. *Hydroxyketone chelators, deferiprone (**HK1**), maltol (**HK3**) and their related compounds (**HK2, 4-8**), were characterized for their cytotoxic profiles against oral human normal and tumor cells. Most hydroxyketones except **HK6** showed relatively higher tumor-specific cytotoxicity. Deferiprone (HK1), which showed the highest tumor specificity, had 10 times higher cytotoxicity than maltol (**HK3**) in both human promyelocytic leukemia HL-60 and human oral squamous cell carcinoma HSC-2 cell lines. The cytotoxic activity of **HK1** against HL-60 and HSC-2 cells was reduced in the presence of $FeCl_3$, while that of **HK3** was significantly increased by $FeCl_3$. Agarose gel electrophoresis showed that **HK1** induced internucleosomal DNA fragmentation in HL-60 cells, but the addition of $FeCl_3$ inhibited the DNA fragmentation. **HK3** did not induce DNA fragmentation in HL-60 cells, regardless of the presence or absence of $FeCl_3$. In HSC-2 cells, **HK1** and **3** did not induce DNA fragmentation in the presence or absence of $FeCl_3$. Colorimetric protease assay showed that **HK1** activated the caspase 3, 8 and 9 in HL-60 cells. On the other hand, **HK3** did not activate the caspase 3, 8 and 9 in HL-60 cells, but activated the caspase 3 only slightly in the presence of $FeCl_3$. **HK1** and **3** also activated the caspase 3, 8 and 9 in HSC-2 cells, but to a lesser extent. The present study suggested that the antitumor activity of hydroxyketones may be modified by Fe^{3+} concentration.*

Tumor cells require high iron concentration for their active proliferation. In the absence of iron, cells are unable to propagate through the cell cycle (1). Therefore, iron is an essential element for tumor cell growth and DNA replication. Previous studies have shown that various iron chelators, such as deferoxamine, deferiprone and hinokitiol, are able to inhibit DNA synthesis and tumor cell proliferation in a number of hepatoma, teratocarcinoma F9 (2, 3) and neuroblastoma cell lines (4). The iron chelators have also been reported to induce apoptosis in proliferating lymphocytes and leukemic cells by iron deprivation (5). Hinokitiol, γ-thujaplicin and β-dolabrin showed strong cytotoxic activities against stomach cancer KATO-III and Ehrlich's ascites carcinoma (6). These molecules have been proved to be suitable ligands for metal ions (Fe^{3+}, Al^{3+}) and have been occasionally used in chelation therapy (7, 8). However, the antitumor mechanisms of iron chelators on tumor cells are not well understood.

In this study, we investigated: (i) whether eight hydroxyketone chelators, such as deferiprone (**HK1**), mimosine (**HK2**), maltol (**HK3**), kojic acid (**HK4**), tropolone (**HK5**), hinokitiol (**HK6**) and 1,2-diketones (**HK7**, **8**) (Figure 1) show tumor-specific cytotoxic activity, using human cultured normal and tumor cells, including two oral carcinoma cell lines; (ii) if so, whether they can induce apoptosis, causing caspase activation and DNA fragmentation as biochemical hallmarks; and (iii) whether their cytotoxic activity is modified by metals.

Materials and Methods

Chemicals. The following chemicals were obtained from each indicated company: 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridone (deferiprone) (MW=139.16) **(HK1)**, mimosine (MW=198.18) **(HK2)**, kojic acid (MW=142.11) **(HK4)**, β-thujaplicin (hinokitiol) (MW=164.20) **(HK6)** and 3-methyl-1,2-cyclopentanedione **(HK7)** (MW=112.13) (Aldrich Chemical Co. Inc, Milwaukee, USA); 3-hydroxy-2-methyl-4-pyrone (maltol) (MW=126.11) **(HK3)**, tropolone (MW=122.11) **(HK5)**, and 1,2-cyclohexanedione (MW=112.13) **(HK8)** (Tokyo Kasei, Co. Ltd, Japan); β-mercaptoethanol (Kanto Chemical Co. Inc. Tokyo, Japan); dithiothreitol (Invitrogen Corp., Carlsbad, CA, USA); Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium and 3-(4,5-

Correspondence to: Prof. Hiroshi Sakagami, Department of Dental Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: (+)81-49-279-2758, 2759 (Lab), Fax: (+)81-49-285-5171, e-mail: sakagami@dent.meikai.ac.jp

Key Words: Deferiprone, maltol, hydroxyketones, cytotoxic activity, oral tumor cells.

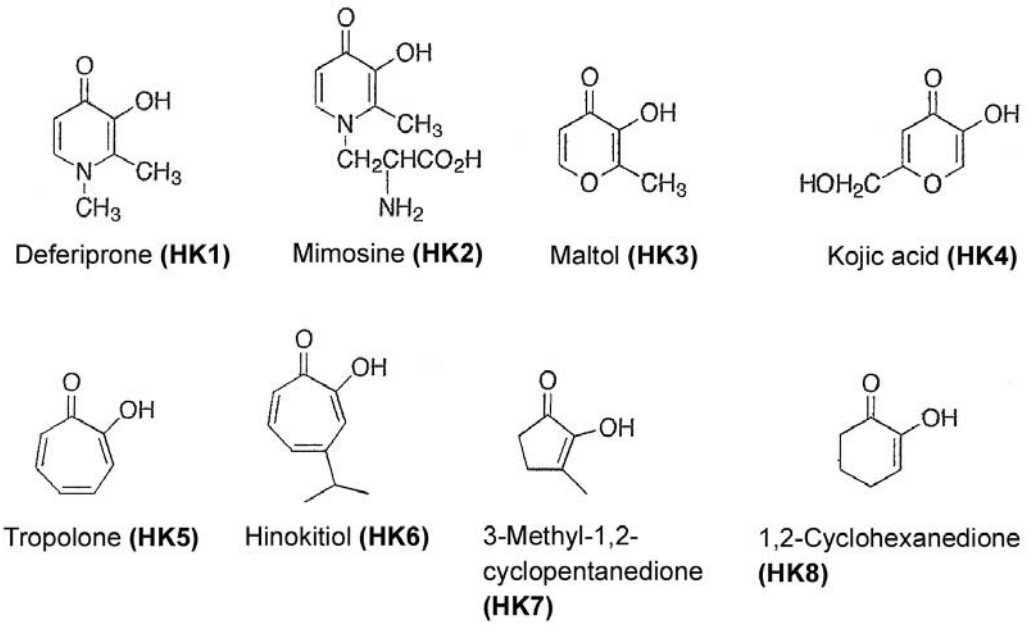

Figure 1. *Chemical structure of hydroxyketones (**HK1-8**).*

dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT)(Sigma Chem. Ind., St. Louis, MO, USA); fetal bovine serum (FBS)(Germini Bio-Products, Woodland, CA, USA); dimethyl sulfoxide (DMSO)(Wako Pure Chem. Ind., Ltd., Osaka, Japan).

Cell culture. Normal cells, human gingival fibroblast (HGF), human pulp cell (HPC) and human periodontal ligament fibroblast (HPLF), were prepared from human periodontal tissue after obtaining informed consent, according to the guidelines of Meikai University Ethic Committee (No. 0206). Since normal cells have a limited life-span (11), cells at 3-7 population doubling level (PDL) were used in the present study. Human oral squamous cell carcinoma (HSC-2, HSC-3) and human submandibular gland carcinoma (HSG) cell lines were kindly provided by Prof. Nagumo, Showa University and Drs. Fukuda and Atsumi, Meikai University, Japan, respectively.

Human promyelocytic leukemia (HL-60) cells were maintained at 37˚C in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Other cells were cultured as a monolayer culture at 37˚C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere, and subcultured by trypsinization.

Cytotoxic activity. The relative viable cell number of adherent cells (HSC-2, HSC-3, HSG, HGF, HPC, HPLF) was determined by MTT methods, while that of non-adherent cells (HL-60) was determined by trypan blue dye exclusion. For MTT assay, near confluent cells in 96-microwell plate (Becton Dickinson Labware, NJ, USA) were treated for 24 hours without (control) or with various concentrations of test samples. The cells were washed once with phosphate-buffered saline without Mg^{2+} nor Ca^{2+} (PBS) and further incubated for 4 hours with 0.2 mg/mL MTT in DMEM + 10% FBS. After removal of the medium, the cells were lysed with 0.1 mL of dimethyl sulfoxide. The absorbance at 540 nm of the solubilized formazan pellet (which reflects the relative viable cell number) was then determined by plate reader. For the trypan blue dye exclusion assay, the number of cells which do not incorporate the trypan blue dye was calculated as viable cell number by hemocytometer. From the dose-response curve, the 50% cytotoxic concentration (CC_{50}) was determined (9).

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). The solution was incubated sequentially with 1 mg/mL RNase A for 1 hour at 50˚C and 1 mg/mL proteinase K for 1 hour at 50˚C. DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with ethanol. DNA was dissolved with DNA loading buffer and then applied to 2% agarose gel electrophoresis. After staining with ethidium bromide, DNA was visualized by UV irradiation (10) and photographed by CCD camera (BioDoc-It™ system, UVP).

Assay for caspase activity. Cells were lysed with 200 μL of lysis solution (MBL). After standing on ice for 10 minutes and centrifugation at 10,000 x g, 4˚C, 5 minutes, the supernatant was collected. To 50 μL sample (equivalent to 0.2 mg protein), 50 μL of 2 x reaction buffer (MBL) containing each substrate for caspase 3 (DEVD-*p*NA (*p*-nitroanilide)), caspase 8 (IETD-*p*NA) or caspase 9 (LEHD-*p*NA), was added. After incubation at 37˚C for 2 hours, the absorbance at 405 nm of *p*NA produced after cleavage of substrates was measured by plate reader.

Results

The structures of the eight hydroxyketone derivatives (**HK1-8**) used in the present study are shown in Figure 1. The

Table I. *Cytotoxic activity of hydroxyketones (**HK1-8**) against cultured human tumor and normal cells.*

Compound	50% Cytotoxic concentration (CC_{50}, μg/mL)						SI[a]= CC_{50} (normal) / CC_{50} (tumor)
	Human tumor cell lines			Normal cells			
	HSC-2	HSC-3	HL-60	HGF	HPC	HPLF	
HK1	13.5	9.9	10.6	>200	>200	>200	>18
HK2	5.7	45.3	24.7	>200	>200	>200	>7.9
HK3	>200	178.1	>200	>200	>200	>200	>><1.0
HK4	20	>200	>200	>200	>200	>200	>><1.4
HK5	25.0	72.7	46.7	>200	185	>200	>4.1
HK6	4.3	19.4	9.6	125.4	113.8	115.8	11
HK7	50.0	>200	>200	>200	>200	>200	>1.3
HK8	37.5	>200	>200	>200	>200	112.5	>><1.2
codeinone	0.78	–	–	6.58	–	–	8.4
A_{540}[b]	0.820			0.494	–	–	

Near confluent cells were incubated for 24 hours without or with various concentrations of each compound, and the relative viable cell number (absorbance at 540 nm of the lysate of MTT-stained cells) was determined by the MTT method. The viable cell number of HL-60 cells was determined by trypan blue exclusion. The CC_{50} was determined from the dose-response curve. Each value represents the mean from duplicate determinations.

[a]determined by the equation: $SI=[(CC_{50})HGF + (CC_{50})HPC + (CC_{50})HPLF / (CC_{50})HSC\text{-}2 + (CC_{50})HSC\text{-}3 + (CC_{50})HL\text{-}60]$.

[b]Absorbance at 540 nm of the lysate of MTT-stained control cells.

estimated 50% cytotoxic concentration (CC_{50} value) for the three tumor cell lines (HSC-2, HSC-3 and HL-60) and three other normal cells (HGF, HPC and HPLF) are summarized in Table I. Cytotoxic data for codeinone, which has α,β-unsaturated ketone, are also included as a control in this Table (11). Most of the hydroxyketones except **HK6** showed relatively low cytotoxic activity against normal cells. On the contrary, however, **HK1**, **2**, **5** and **6** were cytotoxic to HSC-2 and HL-60 cells. Especially, **HK1** showed the highest tumor-specific cytotoxicity (SI>18). Among several tumor cell lines tested, HSG cells were resistant to the cytotoxic effects of **HK1-8** (CC_{50} > 200 μg/mL)(data not shown).

The cytotoxicity of **HK1** against HL-60 and HSG-2 cells was reduced to between one sixth and one tenth by the addition of non-cytototoxic concentrations (0-0.25 mM) of $FeCl_3$ (Figure 2, Table II). However, other metal ions such as Cu^+, Cu^{2+} and Co^{2+} could not reduce the cytotoxicity of **HK1** (Figure 2). It is interesting to note that the cytotoxic activity of all five metal ions was reduced by lower concentrations (0-0.5 mM) of **HK1** (Figure 2), possibly due to the chelating effect of **HK1**. On the other hand, the cytotoxicity of **HK3** against HL-60 and HSC-2 cells was increased by more than 6-7 times in the presence of $FeCl_3$ (Table II).

We investigated whether **HK1** and **3** can induce internucleosomal DNA fragmentation, one of the classic hallmarks of apoptosis, in HL-60 and HSC-2 cells. Figure 3A shows that **HK1** dose-dependently induced DNA fragmentation in HL-60 cells, with an optimal concentration of 25 μg/mL. The fragmentation was, however, abolished by the addition of Fe^{3+}. On the other hand, **HK3** did not induce internucleosomal DNA fragmentation in HL-60 cells, regardless of the presence or absence of Fe^{3+} (Figure 3B). Neither **HK1** nor **3** induced the fragmentation in the presence or absence of Fe^{3+} in HSC-2 cells (data not shown).

HK1 and **3** were also examined to see whether they can activate caspase 3, 8 and 9 in HL-60 and HSC-2 cells. In both HL-60 (Figure 4A) and HSC-2 cells (Figure 5), **HK1** activated caspase 3 in a dose-dependent manner, with an optimal concentration of 100 μg/mL. The addition of Fe^{3+}, however, abolished the activation of caspase 3. **HK1** also activated caspase 8 and caspase 9 to similar extents, and Fe^{3+} again inhibited the stimulation effect of **HK1** (Figure 4A, Figure 5). On the other hand, **HK3** did not activate caspases 3, 8 and 9, although it induced the activation of caspase 3 in the presence of Fe^{3+} in both of HK-60 and HSC-2 cells (Figure 4B, Figure 5).

Discussion

The skeletons of four hydroxyketone chelators (**HK1-4**), with one nitrogen or oxygen heteroatom at the six-membered ring, are almost superimposable. It has been known that these hydroxyketones are capable of chelating metal ion, in particular ferric ion with high affinity (12). **HK1** is a synthetic compound which was initially introduced as an oral ion chelator, for clinical use (marketed by Apotex, Inc. as Ferriprox™). **HK1** inhibits the growth and induces the apoptosis of Kaposi's sarcoma cells (13) or human cervical carcinoma (14). **HK3**, a product of carbohydrate degradation, is known to have weak mutagenic

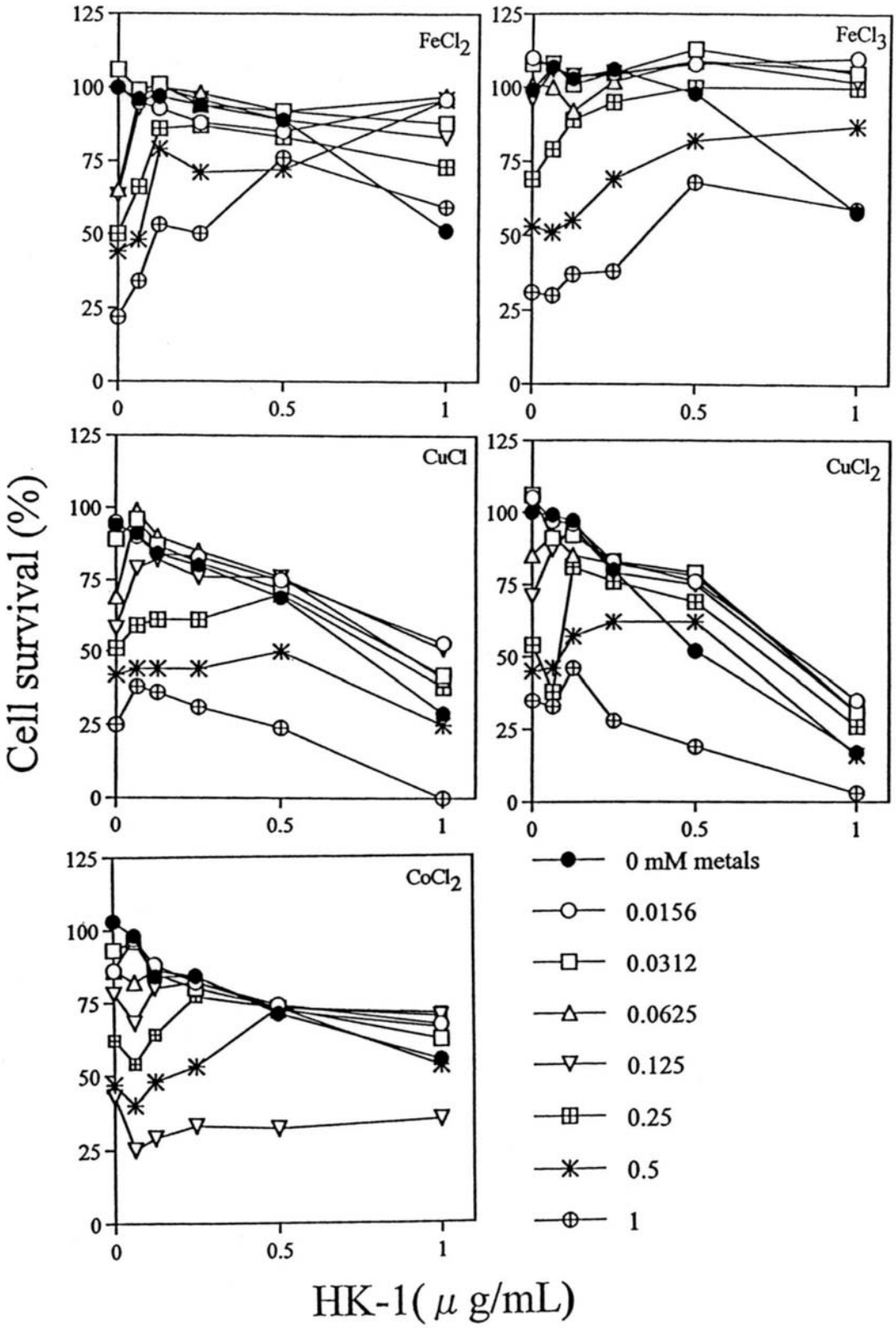

Figure 2. *Effect of metals on the cytotoxicity of **HK1** against **HL-60** cells. HL-60 cells were incubated for 24 hours with the indicated concentrations of HK1 in the absence (●) or presence of 0.0156 (○), 0.0312 (□), 0.0625 (△), 0.125 (▽), 0.25 (⊞), 0.5 (✳) or 1 (⊕) mM each metal, and the viable cell number was determined. Each value represents mean from 4 determinations.*

Table II. *Effects of Fe^{3+} on the cytotoxicity of **HK1** and **3** against HSC-2 and HL-60 cells.*

Compound	Addition of 0.25mM	50% cytotoxic concentration (CC_{50}, μg/mL)	
		HSC-2	HL-60
HK1	–	13.5	11.8
	+	70.0	103
HK3	–	>200	>200
	+	34.2	26.7
A_{540}[a]		0.903±0.0220	(35.9±4.9)x10^4/mL
	+	0.960±0.004	(37.8±4.6)x10^4/mL

[a]Absorbance at 540 nm of the lysate of MTT-stained control cells.

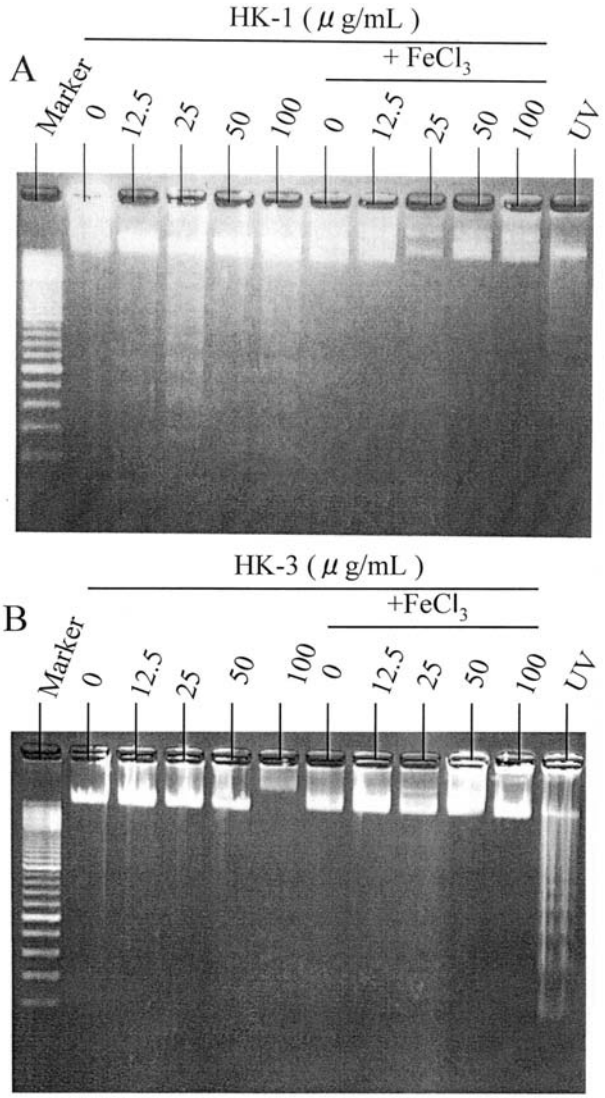

Figure 3. *Induction of DNA fragmentation by **HK1** and **3** in HL-60 cells. HL-60 cells were inoculated at 5 x 10^5 cells/1 mL in 24-well plate, in fresh culture medium (RPMI1640 + 10% FBS) with the indicated concentrations of **HK1** (A) or **3** (B) in the absence or presence of 0.25 mM $FeCl_3$. After incubation for 6 hours, DNA was extracted and applied to agarose gel electrophoresis. Marker, 100 base ladder marker; UV, DNA from apoptotic HL-60 cells induced by UV irradiation (ref. 24).*

activity and to enhance aluminium toxicity in neuronal cells (15). **HK3** was toxic in a dose-dependent manner in neuroblastoma cell lines and primary murine fetal hippocampal neurons, in which the toxic effect is suggested to be mediated through apoptosis (15). **HK4** is a secondary fungal metabolite and recognized for its effective inhibition of tyrosinase in cosmetics (16). **HK4** exerts a promoting effect on thyroid carcinogenesis and causes DNA breaks (17).

It has been reported that **HK1** (5, 18), **HK3** (15) and **HK5** (6, 19) showed potent cytotoxic activity in human hepatoblastoma cell line HepG2, HL-60 or neuroblastoma cell lines. **HK5** and **HK6** showed strong cytotoxic effects *in vitro* on the growth of murine and human tumor cell lines (6, 20). **HK6** has been reported to induce apoptosis of tetratocarcinoma F9 cells through the activation of caspase 3 (3). It was interesting, therefore, to check as to whether such chelators could also display cytotoxicity against oral human tumor cells.

In this study, **HK1** and **HK6** were found to show tumor-specific cytotoxicity against HSC-2 and HL-60 cells (Table I). However, $FeCl_3$ diminished the cytotoxicity of **HK1** (Table II), blocked the **HK1**-induced DNA fragmentation and abrogated the activation of caspase 3 in both HL-60 (Figures 3 and 4) and HSC-2 cells (Figure 5). It has been reported (5) that the presence of Fe^{3+} could abolish the cytotoxicity of iron chelators. This was also verified by the present study.

On the other hand, **HK3** did not show cytotoxicity against HSC-2 and HL-60 cells, although the cytotoxicity of **HK3** against HL-60 and HSC-2 cells was significantly increased by the addition of Fe^{3+} (Table II). **HK3** is relatively non-toxic to cancer cells (21, 22). However, the cytotoxicity of **HK3** was enhanced in the presence of Fe^{3+}, possibly by a chelation with Fe^{3+}. These data suggest that the antitumor activity of hydroxyketones may be modified by Fe^{3+} concentration.

Few investigators have reported on tumor-specific cytotoxic activity, although this index (SI values) is very important to explore new anticancer agents. Any apoptosis inducers should lose their clinical value, if they have small SI values. We found that among eight hydroxyketones, **HK1** (SI>18), **HK2** (SI>7.9), **HK5** (SI>4.1) and **HK6** (SI=11) showed relatively high tumor-specific cytotoxicity, while others did not show such effects (Table I). Among these, **HK1** showed the highest tumor-specificity. However, the sensitivity against **HK1** differed considerably from cell to cell. This point should be pursued, using many types of tumor cells.

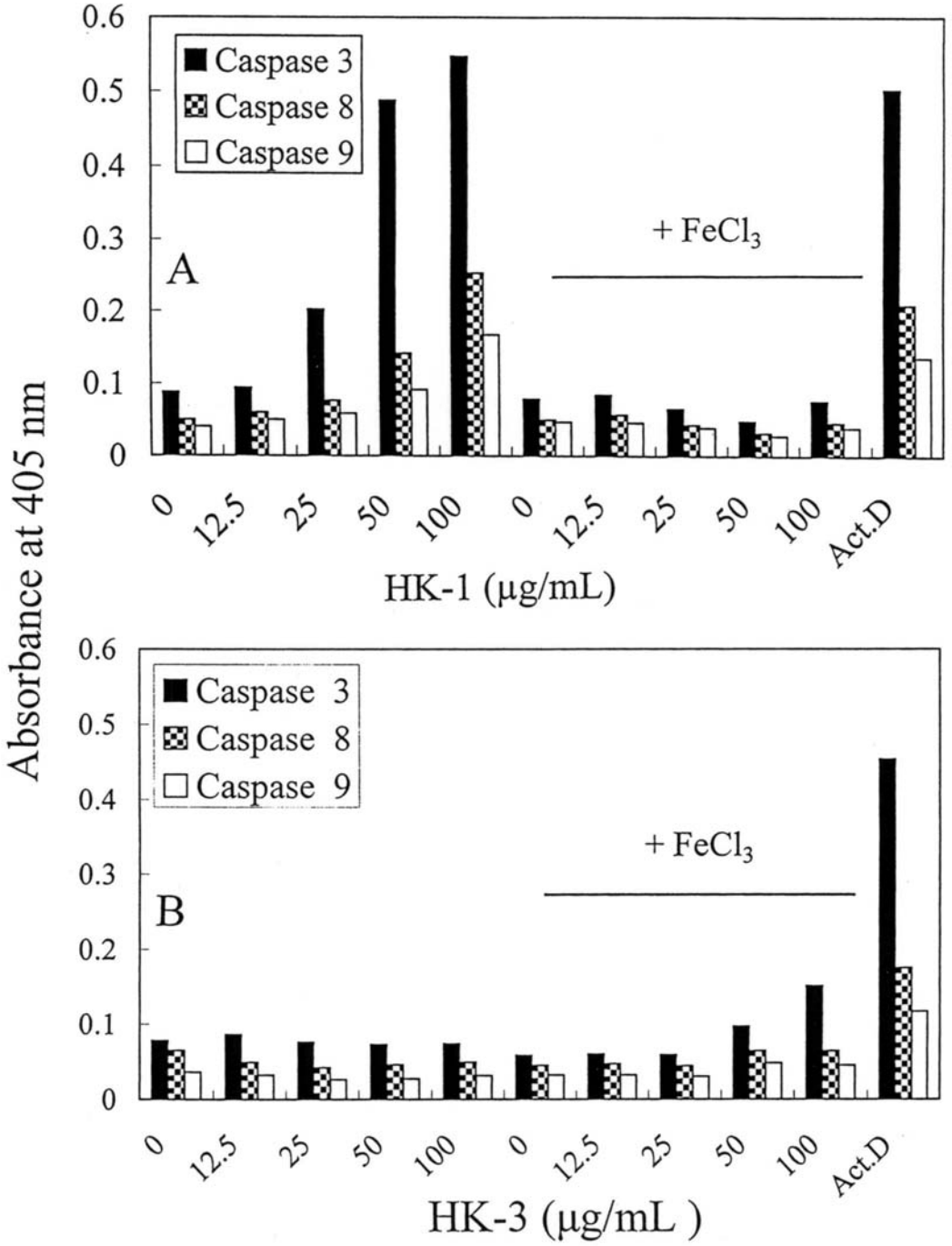

Figure 4. *Activation of caspases 3, 8 and 9 by **HK1** and **3** in HL-60 cells. HL-60 cells were incubated for 4 hours with the indicated concentrations of **HK1** (A) or **3** (B) in the absence or presence of 0.25 mM $FeCl_3$ and then caspases 3, 8, 9 activities were assayed. Actinomycin D, positive control (1 μg/mL actinomycin D-treated HL-60 cells).*

Recent studies on the mechanism of cell death induction by the iron chelator, deferoxamine, revealed that p38 MAP kinase plays an important role in iron chelator-mediated cell death in HL-60 cells by activating the downstream apoptotic cascade that executes the cell death pathway (23). In this study, **HK1** activated caspase 3, *via* both the caspase 9-involved mitochondria-mediated intrinsic pathway and the caspase 8-involved non-mitochondria-mediated extrinsic pathway, finally leading to internucleosomal DNA fragmentation in HL-60 cells

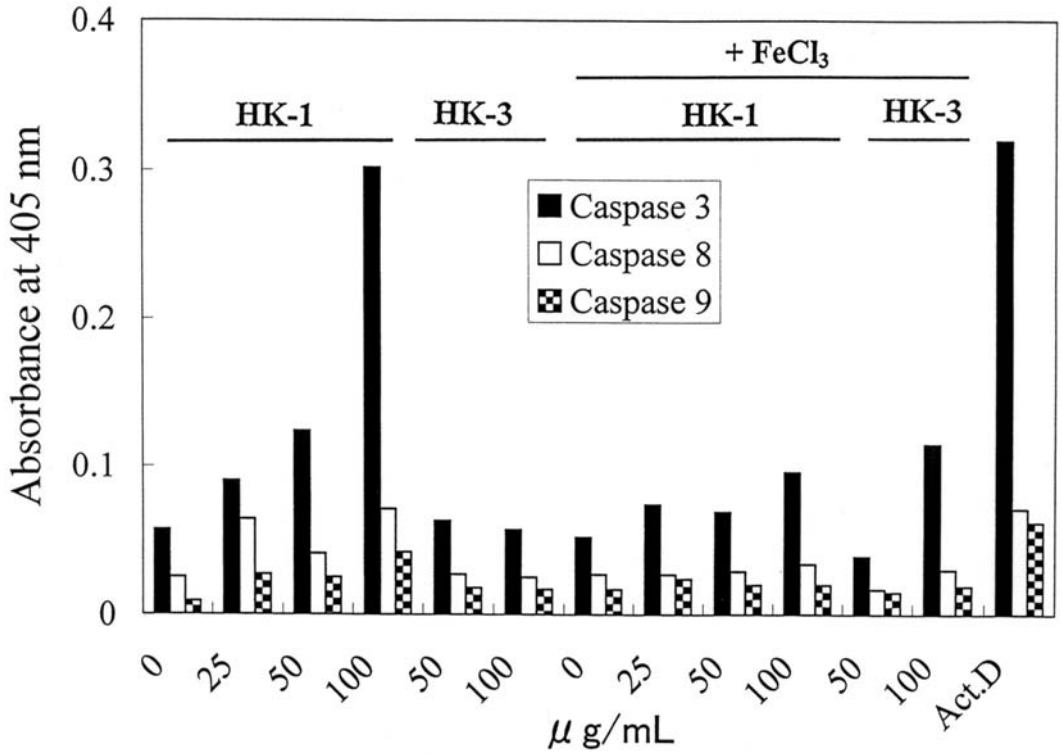

Figure 5. *Activation of caspases 3, 8 and 9 by **HK1** and **3** in HSC-2 cells. HSC-2 cells were incubated for 4 hours with the indicated concentrations of **HK1** or **3** in the absence or presence of 0.25 mM $FeCl_3$ and then caspases 3, 8, 9 activities were assayed. Actinomycin D, positive control (1 μg/mL actinomycin D-treated HSC-2 cells).*

(24). We also found that the activation of caspase 3, 8 and 9 did not always induce internucleosomal DNA fragmentation, as observed in HSC-2 cells. The activation of caspase above a certain threshold might be required for the induction of DNA fragmentation. Further studies are required to elucidate the molecular mechanism of apoptosis induction by hydroxyketones.

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