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

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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 FeCl3, while that of HK3 was significantly increased by FeCl3. Agarose gel electrophoresis showed that HK1 induced internucleosomal DNA fragmentation in HL-60 cells, but the addition of FeCl3 inhibited the DNA fragmentation. HK3 did not induce DNA fragmentation in HL-60 cells, regardless of the presence or absence of FeCl3. In HSC-2 cells, HK1 and 3 did not induce DNA fragmentation in the presence or absence of FeCl3. 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 FeCl3. 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 Fe3+ 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 defereroxamine, 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 ascesis carcinoma (6). These molecules have been proved to be suitable ligands for metal ions (Fe3+, Al3+) 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(1H)-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-cyclohexanedicione (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-
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 lifespan (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% CO2 atmosphere. Other cells were cultured as a monolayer culture at 37°C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO2 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 Mg2+ nor Ca2+ (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 (CC50) was determined (9).

**Assay for DNA fragmentation.** Cells were lysed with 50 µL lystate 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-pNA (p-nitroanilide)), caspase 8 (IETD-pNA) or caspase 9 (LEHD-pNA), was added. After incubation at 37°C for 2 hours, the absorbance at 405 nm of pNA 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>50% Cytotoxic concentration (CC50, µg/mL)</th>
<th>SIa</th>
<th>SFb</th>
</tr>
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<tr>
<td></td>
<td>Human tumor cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSC-2</td>
<td>HSC-3</td>
<td>HL-60</td>
</tr>
<tr>
<td>HK1</td>
<td>13.5</td>
<td>9.9</td>
<td>10.6</td>
</tr>
<tr>
<td>HK2</td>
<td>5.7</td>
<td>45.3</td>
<td>24.7</td>
</tr>
<tr>
<td>HK3</td>
<td>&gt;200</td>
<td>178.1</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HK4</td>
<td>20</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HK5</td>
<td>25.0</td>
<td>72.7</td>
<td>46.7</td>
</tr>
<tr>
<td>HK6</td>
<td>4.3</td>
<td>19.4</td>
<td>9.6</td>
</tr>
<tr>
<td>HK7</td>
<td>50.0</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HK8</td>
<td>37.5</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>codeinone</td>
<td>0.78</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A540b</td>
<td>0.820</td>
<td>0.494</td>
<td>–</td>
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</table>

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 CC50 was determined from the dose-response curve. Each value represents the mean from duplicate determinations.

a determined by the equation: SI=[(CC50)HGF + (CC50)HPC + (CC50)HPLF / (CC50)HSC-2 + (CC50)HSC-3 + (CC50)HL-60].

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

estimated 50% cytotoxic concentration (CC50 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 (CC50 > 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-cytotoxic concentrations (0-0.25 mM) of FeCl3 (Figure 2). However, HK1 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 HSG-2 cells was increased by more than 6-7 times in the presence of FeCl3.

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 Fe3+. On the other hand, HK3 did not induce internucleosomal DNA fragmentation in HL-60 cells, regardless of the presence or absence of Fe3+ (Figure 3B). Neither HK1 nor 3 induced the fragmentation in the presence or absence of Fe3+ in HSC-2 cells (data not shown).

HK1 and 3 were also examined to see whether they can activate caspases 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 Fe3+, however, abolished the activation of caspase 3. HK1 also activated caspase 8 and caspase 9 to similar extents, and Fe3+ 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 Fe3+ 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.
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, FeCl3 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 Fe3+ 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 Fe3+ (Table II). HK3 is relatively non-toxic to cancer cells (21, 22). However, the cytotoxicity of HK3 was enhanced in the presence of Fe3+, possibly by a chelation with Fe3+. These data suggest that the antitumor activity of hydroxyketones may be modified by Fe3+ 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.

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Table II. Effects of Fe3+ on the cytotoxicity of HK1 and 3 against HSC-2 and HL-60 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Addition of 0.25mM</th>
<th>50% cytotoxic concentration (CC50 ìg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HSC-2</td>
</tr>
<tr>
<td>HK1</td>
<td>–</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>70.0</td>
</tr>
<tr>
<td>HK3</td>
<td>–</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>34.2</td>
</tr>
<tr>
<td>HK4</td>
<td>–</td>
<td>0.903±0.0220 (35.9±4.9)x10³/mL</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.960±0.004 (37.8±4.6)x10³/mL</td>
</tr>
</tbody>
</table>

*Absorbance at 540 nm of the lysate of MTT-stained control cells.

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Figure 3. Induction of DNA fragmentation by HK1 and 3 in HL-60 cells. HL-60 cells were inoculated at 5 x 10⁵ 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 FeCl3. 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).
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.
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.

References


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 FeCl3 and then caspases 3, 8, 9 activities were assayed. Actinomycin D, positive control (1 μg/mL actinomycin D-treated HSC-2 cells).


Received October 21, 2003
Accepted January 29, 2004