Induction of Apoptosis by β-Diketones in Human Tumor Cells

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Abstract. A variety of β-diketones were evaluated for their cytotoxic profiles against human normal and tumor cells. Among 22 compounds (BD1-22) tested, the cytotoxicity of 3-formylchromone (BD17) (CC50=7.8 µg/mL) against human oral squamous cell carcinoma (HSC-2) cells was higher than that of curcumin (CC50=23.6 µg/mL). Tumor cell-specific cytotoxicity was also detected in BD17 which exhibited little cytotoxic activity against a normal human cell, gingival fibroblast (HGF). (+)-3-(Trifluoroacetyl)camphor (BD12) (CC50=29.7 µg/mL) are enantiomers and showed cytotoxicity comparable to curcumin and dibenzoylmethane (BD2) (CC50=22.5 µg/mL). BD13 did not induce DNA fragmentation in HL-60 cells nor activate caspase 3, 8 and 9 in both HL-60 and HSC-2 cells, regardless of the presence or absence of FeCl3. On the other hand, BD17 was found to induce apoptosis in HSC-2 and HL-60 cells, as judged by internucleosomal DNA fragmentation, caspase 3, 8 and 9 activation and dysfunction of mitochondrial membrane potential. The cytotoxic activity of BD13, BD17 and curcumin was significantly reduced by chelation with FeCl3. The tumor-specific cytotoxicity and apoptosis-inducing activity of BD17 against human tumor cells undoubtedly warrant further studies of its efficacy as a cancer chemotherapeutic agent.

Curcumin, a dietary pigment from Curcuma longa L., has various biological activities, including antioxidant, cancer preventive and antiangiogenesis activity (1-9). The structure-activity relationship studies have been discussed in several papers; in most of them the biological activity is attributed to the presence of a β-diketone moiety (10, 11). 1,3-Diphenyl-1,3-propanedione (dibenzoylemethane), a minor β-diketone constituent of licorice and sunscreens, has also been shown to exhibit anti-neoplastic effects in several animal models (12-14). Structurally, β-diketones would exist mainly in the enolic form and form metal chelates in the presence of various ions, such as ferric ions (Fe2+), copper ions (Cu2+) and zinc ions (Zn2+). Recent studies suggest that metal chelates induce apoptotic cell death in various tumor cell lines and are potent antitumor agents for tumor cells including malignant melanomas (15, 16). Thus, the properties of β-diketones as chelators of metal ions may play an important role in various biological functions of the compound, especially with antitumor activity.

These findings urged us to evaluate the cytotoxic activity of 22 β-diketones (BD1-22) against human cultured tumor and normal cells in order to search for more potent and selective cytotoxic antitumor agents.

Materials and Methods

Chemicals. The following chemicals were obtained from each indicated company: 1-benzoylacetone (MW=164)(BD1), dibenzoylmethane (MW=224)(BD2), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (MW=216)(BD3), thienoyltrifluoroacetone (MW=222)(BD5), 2,4-pentanedione (MW=100)(BD6), 1,1,1-trifluoro-2,4-pentanedione (MW=154)(BD7), hexafluoroacetacetone (MW=208)(BD8), 3-chloro-2,4-pentanedione (MW=134.5)(BD9), 2,6-dimethyl-3,5-heptanedione (MW=150)(BD11), 1,3-cyclopentanediene (MW=98)(BD18), 2-ethyl-1,3-cyclopentanediene (MW=126)(BD19), 1,3-cyclohexanediene (MW=112)(BD20), 2-phenyl-1,3-indandione (MW=222)(BD22), curcumin (MW=368) and gallic acid (MW=158) (Tokyo Kasei Co Ltd, Tokyo, Japan); 4,4,4-trifluoro-1-(2-furyl)-1,3-butanedione (MW=206)(BD4), 3-ethyl-2,4-pentanedione (MW=128)(BD10), (+)-3-(trifluoroacetyl)camphor (MW=248)(BD12), (-)-3-(trifluoroacetyl)camphor (MW=248)(BD13), 2-acetylcyclopentanone (MW=126)(BD14), 2-acetylcyclobutanone (MW=140)(BD15), 2-acetylcyclopentanone (MW=188)(BD16), 3-formylchromone (MW=174)(BD17) and 1,3-indandione (MW=146)(BD21) (Aldrich Chemical Co, Inc, Milwaukee, USA); Dulbecco’s Modified Eagle Medium (DMEM), RPMI1640 medium and 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT)(Sigma Chem. Ind., St.

Key Words: β-Diketone, apoptosis, cytotoxic activity, oral tumor cells.
Louis, MO, USA); fetal bovine serum (FBS)(Gemini 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 obtained from human periodontal tissue after informed consent, according to the guideline of Meikai University Ethics Committee, Japan, (No. 0206). Since normal cells have a limited life-span (11), cells at 3-7 population doubling level (PDL) were used for the present study. The human oral squamous cell carcinoma cell lines (HSC-2, HSC-3) were supplied by Prof. Nagumo, Showa University, and Dr. Fukuda, Meikai University, Japan, respectively. The human submandibular gland carcinoma cell line (HSG) was supplied by Drs. Atsumi and Kurihara, Meikai University. The human promyelocytic leukemia cell line (HL-60) was supplied by Prof. Nakaya, Showa University. 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 was determined by MTT methods, while that of non-adherent cells (HL-60 cells) was determined by trypan blue dye exclusion. For MT1 assay, the cells 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+ or 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 microplate reader (Biochromatic Labsystem, Helsinki, Finland). 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 (17). Tumor-specific cytotoxicity (SI value) was determined by the following equation: SI = CC50 (HSG)/CC50 (HSC-2).

Assay for DNA fragmentation. Cells were lysed with 50 μL lysis 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 and photographed by CCD camera (Bio Doc Inc, UVP) (18).
**Assay for mitochondrial membrane potential.** After treatment with BD17, cells were incubated at 37°C in a 5% CO₂ incubator for 3, 5 and 8 hours, respectively. The cells were stained for 20 minutes with a MitoCapture, a mitochondrial membrane potential indicator (Bio Vision, Mountain View, CA, USA). Then they were observed under a fluorescence Microscope (Olympus IX70), using a band-pass filter that detects FITC and Rhodamine.

**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 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 4 hours, the absorbance at 405 nm of pNA produced after cleavage of substrates was measured by microplate reader.

**Results**

**Cytotoxic activity.** Twenty-two β-diketones [BD1-22] (structures shown in Figure 1) were first subjected to the screening test of cytotoxic activity against HSC-2 and HGF cells. 3-Formylchromone (BD17) (CC₅₀=7.8 μg/mL) showed the highest cytotoxic activity against HSC-2 cells, followed by (-)-3-(trifluoroacetyl)camphor (BD13) (CC₅₀=21.4 μg/mL), its enantiomer (+)-3-(trifluoroacetyl)camphor (BD12) (CC₅₀=29.7 μg/mL) and 4,4,4-trifluoro-1-phenyl-1,3-butanedione (BD3) (CC₅₀=66.0 μg/mL) (Table I). Cytotoxic data for curcumin (CC₅₀=23.6 μg/mL), dibenzoylmethane (BD2) (CC₅₀=22.5 μg/mL) and gallic acid (CC₅₀=59.3 μg/mL) are also included as a control in Table I. Furthermore, both BD13 and 17 showed the highest tumor-specific cytotoxicity (SI value=CC₅₀).

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**Table I. Cytotoxic activity of β-diketones (BD1-22) against cultured human tumor and normal cells.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>HSC-2</th>
<th>HSC-3</th>
<th>HSG</th>
<th>HL-60</th>
<th>HGF</th>
<th>HPC</th>
<th>HPLF</th>
<th>SI a</th>
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<td>–</td>
<td>162</td>
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<tr>
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<td>26.8</td>
<td>51.4</td>
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<td>82.8</td>
<td>55.6</td>
<td>4309</td>
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<td>136</td>
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<td>–</td>
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<td>–</td>
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<td>–</td>
<td>&gt;200</td>
<td>–</td>
<td>–</td>
<td>&lt;1.0</td>
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<td>–</td>
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<td>–</td>
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<td>–</td>
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<td>18.6</td>
<td>170</td>
<td>148</td>
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<td>–</td>
<td>–</td>
<td>65.6</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>BD21</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>&gt;200</td>
<td>–</td>
<td>–</td>
<td>&gt;1.1</td>
</tr>
<tr>
<td>BD22</td>
<td>165</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&gt;200</td>
<td>–</td>
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<tr>
<td>curcumin</td>
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<tr>
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<td>–</td>
<td>77.5</td>
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<td>–</td>
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A₅₄₀ 0.304 0.304 0.131 1.28 x 10⁶/mL

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)HSC-2
Figure 2. Effect of FeCl₃ on the cytotoxicity of BD13, BD17 and curcumin against HSC-2 cells. HSC-2 cells were incubated for 24 hours with the indicated concentrations of BD13, BD17 or curcumin in the presence (●) or absence of 0.25 mM FeCl₃ (●●), and the viable cell number was determined by MTT method. Each value represents the mean from 4 determinations.

Figure 3. Induction of DNA fragmentation by BD13, BD17 and curcumin 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 each compound in the absence or presence of 0.25 mM FeCl₃. After incubation of 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 (23).
(HGF)/CC50 (HSC-2)=5.4 and 4.3, respectively) and were more or equally potent than that of curcumin (SI=1.7) and gallic acid (SI=1.3). The highly selective tumor-specific cytotoxicity of these compounds was confirmed by evaluating their cytotoxicity against another three tumor cells (HSC-3, HSG and HL-60) and two normal cells (HPC and HPLF) (Table I).

The cytotoxic activity of BD13 and 17 against HSC-2 was reduced to 1/2 - 1/3 by the addition of FeCl₃ (0.25 mM), while FeCl₃ more potently reduced the cytotoxic activity of curcumin (Figure 2).

Agarose gel electrophoresis showed that BD17 weakly induced internucleosomal DNA fragmentation in HL-60 cells, regardless of the absence or presence of FeCl₃ (0.25 mM) (Figure 3). BD13 did not induce DNA fragmentation in HL-60 cells, regardless of the presence or absence of FeCl₃.

Curcumin also only marginally induced internucleosomal DNA fragmentation (Figure 3).

We next investigated the possible change in the mitochondrial membrane potential, using green fluorescence dye. Figure 4 shows that the number of cells stained with green fluorescence dye, expressing the dysfunction of mitochondrial membrane potential, was increased at 8 hours after treatment with BD17. This increase suggests the occurrence of apoptosis in these cells (Figure 4).

We investigated whether BD17, 13 and curcumin can activate caspase 3, 8 and 9, executioners of apoptosis, in HL-60 and HSC-2 cells. Colorimetric protease assay showed that BD17 and curcumin activated caspase 3, 8 and 9 in a dose-dependent manner, more profoundly in HL-60 cells than in HSC-2 cells (Figure 5). The addition of FeCl₃ slightly reduced the activation of caspase 3, 8 and 9 induced by BD17 in HL-60 cells, but more prominently than induced by curcumin (Figure 5). On the other hand, BD13 did not activate caspases 3, 8 and 9 in HL-60 and HSC-2 cells, regardless of the presence or absence of FeCl₃, consistent with the lack of DNA fragmentation.

**Discussion**

Recently, many studies have demonstrated the remarkable cancer preventive properties of curcumin (3, 4, 8, 9). Curcumin exhibited remarkable cytotoxic effects on various cancer cells (3, 4, 6, 7, 9) and induced apoptotic cell death in human promyelocytic leukemia HL-60 cells and human oral squamous cell carcinoma HSC-4 cells (1). The apoptosis-inducing activity of curcumin is shown to be dose- and time-dependent (6). An early DNA fragmentation is seen at 4 hours after exposure to 3.5 μg/mL curcumin (6). Dibenzoylmethane (BD2) has been...
reported to inhibit growth in chemically-induced skin and mammary tumors (8, 19) and in prostate cancer cell lines (12). In this study, BD2 showed potent cytotoxicity in oral tumor cell lines which was equipotent with curcumin.

The cytotoxic activities of BD12, BD13, BD17 and curcumin against HSG-2 was significantly reduced by the addition of FeCl3 (0.25 mM). It has been reported (15) that the cytotoxicity of iron chelators, deferoxamine, deferiprone and hinokitiol, was diminished by the addition of Fe3+. It is known that both BD12 and 13 are capable of chelating metal ions with high affinity (20, 21). These data suggest that antitumor activity of β-diketones may be modified by the Fe3+ concentration. However, we cannot exclude another possibility that FeCl3 might attach to the cellular surface and prevent the entry of the compounds, especially curcumin.

Figure 5. Activation of caspases 3, 8 and 9 by BD13, BD17 and curcumin in HL-60 and HSC-2 cells. HL-60 and HSC-2 cells were incubated for 4 hours with the indicated concentrations of the compound 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 HL-60 cells).
3-Formylchromone (BD17) is known to be an inhibitor of tyrosine kinase (22), but to the best of our knowledge, other biological activities have not been examined. It should be pointed out that the cytotoxicity depends on the presence of an aldehyde group at position 3 in BD17. However, the role of the aldehyde in cytotoxicity is currently not known. Further studies of the structure and activity relationship are needed. We demonstrated that BD17, which shows the highest tumor-specific cytotoxicity (SI=5.4) among 22 β-diketones, dose-dependently induced apoptosis in HSC-2 and HL-60 cells by caspase 3 activation, interchromosomal DNA fragmentation and dysfunction of mitochondrial membrane potential.

Apoptosis is the principle mechanism employed by chemotherapeutic drugs in eradicating tumor cells. Resistant tumor cells evade the action of anticancer agents by increasing their apoptotic threshold. This has spurred on the development of novel chemical compounds capable of inducing apoptosis in resistant tumor cells. Therefore, the highly potent cytotoxicity and apoptosis-inducing activity of 3-formylchromone (BD17) against human tumor cells undoubtedly warrant further studies of its efficacy as a cancer chemopreventive agent. The present study also demonstrated that β-diketones such as BD13, which showed higher tumor-specific cytotoxicity (SI=4.3), did not induce apoptosis-associated characteristics. This indicates the necessity of elucidating the mechanism of the expression of tumor-specific cytotoxicity, as well as apoptosis.

References


Received December 8, 2003
Accepted February 10, 2004