Cytotoxic Activity of 5-Benzoylimidazole and Related Compounds against Human Oral Tumor Cell Lines

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Abstract. A total of 24 benzoylimidazoles and structurally-related compounds were investigated for their cytotoxic activity against oral tumor cells and normal gingival fibroblast. Compound 23 (5-(2-hydroxybenzoyl)-2-phenylimidazole) showed the highest cytotoxic activity against both human oral tumor cell lines (human squamous cell carcinoma HSC-2, human salivary gland tumor HSG) and normal human gingival fibroblast (HGF). Compounds 7 (2-(2-hydroxybenzoyl)benzimidazole), 14 (1,3-diethyl-5-(2-hydroxybenzoyl)-4-imidazoline-2-thione) and 18 (5-(2-hydroxy-4-methylbenzoyl)-3-methyl-2-methylimidino-4-thiazoline) showed slightly lower cytotoxic activity, but higher tumor-specific cytotoxic action. The cytotoxic activity of compound 23 was significantly reduced by CuCl2, but not by CoCl2, FeCl3, or by antioxidants (N-acetyl-L-cysteine, sodium ascorbate, catalase). Compound 23 did not show any detectable oxidation potential (determined by NO monitor). Agarose gel electrophoresis demonstrated that compound 23 induced DNA fragmentation in human promyelocytic leukemia cells HL-60, but not in HSG cells. These data suggested that the response to compound 23 might be different from cell to cell.

We have recently summarized the efficacy of polyphenols in preventing oral diseases (1). Our strategy include the following three steps: (i) screening of various natural and synthetic compounds, (ii) elucidation of action mechanism and, (iii) interaction with the oral environment. We have recently found that tannins, such as macrocyclic hydrolyzable tannins, epigallocatechin gallate (EGCG) (a major component of green tea), gallic acid (a component unit of tannin) (2-4) and isoprenylated flavonoids (5, 6) induced apoptotic cell death, characterized by DNA fragmentation (identified by TUNEL method and agarose gel electrophoresis) and caspase activation (identified by M30 monovalonal antibody), in human oral tumor cells. Lignins, which had little cytotoxic activity, synergistically stimulated the cytotoxic activity of sodium ascorbate (7). We have recently reported that newly synthesized benzoxepin/ benzothiepin induced tumor-specific cytotoxicity and inter-nucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, in oral tumor cell lines (8). These findings prompted us to initiate the structure-activity relationship of various synthetic compounds, for their clinical application.

Imidazol(ine) derivatives inhibit the acetylcholine-induced secretion of catecholamines in adrenal chromaffin cells (9) by blocking nicotinic acetylcholine receptors (10). These drugs were previously reported to act as α1-adrenoceptor antagonists. Also, imidazol(ine) derivatives have been shown to be neuroprotective in brain injuries of necrotic (11) and apoptotic neuronal cell death (12). Previously, the neuroprotective effects of imidazolines against N-methyl-D-aspartate (NMDA)-induced neurotoxicity in cultured cerebellar granule cells (13,14) have been reported. These imidazol(ine) derivatives possibly show the broad spectrum of biological effects on primary or permanent cells apart from the cells described above. We report here the relative potency of 5-benzoylimidazole and related compounds (15) to induce cytotoxicity against human oral squamous cell carcinoma cells HSC-2 and human salivary gland tumor cells HSG, in comparison with human normal gingival fibroblast HGF and human promyelocyte leukemia HL-60 cells.

Materials and Methods

Materials. The following reagents were obtained from the indicated companies: Dulbecco’s modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Gland Island, NY, USA); fetal bovine serum (FBS)(JRH Bioso, Lenexa, KS, USA); CoCl2·6H2O, FeCl3·6H2O, CaCl2·2H2O, dimethyl sulfoxide (DMSO)(Wako Pure Chem. Ind. Ltd., Osaka, Japan), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium

Key Words: 5-Benzoylimidazole, cytotoxic activity, oral tumor cells, DNA fragmentation.
bromide (MIT), catalase, N-acetyl-L-cysteine (NAC) (Sigma Chemical Ind. St. Louis, MO, USA); RNase A, proteinase K (Boehringer Mannheim, Germany).

Synthesis of 3-azolylthio-4H-1-benzopyran-4-one and 2-benzoxylimidazo[2,1-b]thiazole (general procedure A). A mixture of 3-iodochrome (136 mg, 0.5 mmol), mercaptoazoles (0.5 mmol) and K2CO3 (276 mg, 2 mmol) in DMF (5 mL) was stirred for 1.4 hours at room temperature. After removal of the K2CO3, the reaction mixture was diluted with water and extracted with CHCl3. The organic layer was dried over Na2SO4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (hexane-AcOEt).

Synthesis of 3-(1H-benzimidazol-2-ylthio)-4H-1-benzopyran-4-one (1) and 2-(2-hydroxybenzoyl)benzimidazol[2,1-b]thiazole (7). According to the general procedure A, 3-iodochrome (136 mg, 0.5 mmol) and 2-mercaptobenzimidazole (75 mg, 0.5 mmol) were treated with K2CO3 for 1 hour to give 1 (41 mg, 28%) and 7 (88 mg, 60%), respectively.

Synthesis of 3-(1H-benzimidazol-2-ylthio)-6-methoxy-4H-1-benzopyran-4-one (2). According to the general procedure A, 3-ido-6-methoxychromone (151 mg, 0.5 mmol) and 2-mercaptobenzimidazole (75 mg, 0.5 mmol) were treated with K2CO3 for 4 h to give 2 (139 mg, 86%).

Synthesis of 3-(1H-imidazol-2-ylthio)-4H-1-benzopyran-4-one (3) and 2-(2-hydroxybenzoyl)imidazol[2,1-b]thiazole (10). According to the general procedure A, 3-iodochrome (136 mg, 0.5 mmol) and 2-mercaptobenzimidazole (50 mg, 0.5 mmol) were treated with K2CO3 for 1 hour to give 3 (22 mg, 18%) and 10 (76 mg, 62%), respectively.

Synthesis of 3-(1-methyl-1H-imidazol-2-ylthio)-4H-1-benzopyran-4-one (4). According to the general procedure A, 3-iodochrome (136 mg, 0.5 mmol) and 2-mercaptop-1-methylimidazole (57 mg, 0.5 mmol) were treated with K2CO3 for 1 hour to give 4 (114 mg, 88%).

Synthesis of 3-(1H,1,2,4-triazol-3-ylthio)-4H-1-benzopyran-4-one (5). According to the general procedure A, 3-iodochrome (136 mg, 0.5 mmol) and 2-mercapto-1,2,4-triazole (51 mg, 0.5 mmol) were treated with K2CO3 for 2 hours to give 5 (88 mg, 72%).

Synthesis of 3-(4-methyl-4H-1,2,4-triazol-3-ylthio)-4H-1-benzopyran-4-one (6). According to the general procedure A, 3-iodochrome (136 mg, 0.5 mmol) and 2-mercapto-4-methyl-1,2,4-triazole (58 mg, 0.5 mmol) were treated with K2CO3 for 2 hours to give 6 (91 mg, 70%).

Synthesis of 2-(2-hydroxybenzoyl)-7-methoxybenzimidazol[2,1-b]thiazole (8). According to the general procedure A, 3-iodochrome (136 mg, 0.5 mmol) and 2-mercaptop-5-methoxybenzimidazole (90 mg, 0.5 mmol) were treated with K2CO3 for 1 hour to give 8 (73 mg, 45%).

Synthesis of 5H-benzimidazol[2',1':2,1']thiazolo[4,5-b]benzopyran-5-one (9). To a stirred solution of 7 (84 mg, 0.3 mmol) and DBU (182 mg, 1.2 mmol) in CH2Cl2 (3 mL), a solution of iodine (84 mg, 0.33 mmol) in CH2Cl2 (2 mL) was drop-wise added over a 20 minutes period at 0°C. After being stirred for 10 minutes, the reaction was quenched at the same temperature by adding saturated aqueous Na2S2O3 (2 mL). Then the mixture was extracted with CH2Cl2, the combined organic layers were dried over Na2SO4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give 9 (81 mg, 92%).

Synthesis of 3-benzoyl-4-imidazoline-2-thione and 3-benzoyl-2-imino-4-thiazoline (general procedure B). A mixture of 3-iodochrome (136 mg, 0.5 mmol), thiourea (2 mmol), Bu4NCl (139 mg, 0.5 mmol) and K2CO3 (690 mg, 5 mmol) in benzene (15 mL) was refluxed for 10-18 hours. After removal of K2CO3, the reaction mixture was diluted with water and extracted with CHCl3. The organic layer was dried over Na2SO4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (hexane-AcOEt).
Figure 1. Structure of 3-azolylthio-4H-1-benzopyran-4-one (1-6), 2-benzoylimidazo[2,1-b]thiazole (7-10), 5-benzoyl-4-imidazoline-2-thione (11-15), 5-benzoyl-2-imino-4-thiazoline (16-19) and 5-benzoylimidazole (20-24).
Table I. Cytotoxic activity and tumor-specificity of 5-benzoylimidazole and related compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxic activity (CC50: µg/mL)</th>
<th>Tumor specificity C/A C/B</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HSC-2 (A)</td>
<td>HSG (B)</td>
</tr>
<tr>
<td>3-azolyl-4H-1-benzopyran-4-one</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>76</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>174</td>
<td>221</td>
</tr>
<tr>
<td>4</td>
<td>&gt;400</td>
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<tr>
<td>5</td>
<td>225</td>
<td>218</td>
</tr>
<tr>
<td>6</td>
<td>296</td>
<td>330</td>
</tr>
<tr>
<td>2-benzoylimidazo[2,1-b]thiazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>44</td>
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<tr>
<td>8</td>
<td>&gt;400</td>
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<td>9</td>
<td>164</td>
<td>264</td>
</tr>
<tr>
<td>10</td>
<td>152</td>
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<tr>
<td>5-benzoyl-4-imidazoline-2-thione</td>
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<tr>
<td>11</td>
<td>104</td>
<td>128</td>
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<tr>
<td>12</td>
<td>75</td>
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</tr>
<tr>
<td>15</td>
<td>&gt;400</td>
<td>310</td>
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<td>5-benzoyl-2-imino-4-thiazoline</td>
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<td>87</td>
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<tr>
<td>5-benzoylimidazole</td>
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<td></td>
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<tr>
<td>20</td>
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<td>28</td>
</tr>
<tr>
<td>24</td>
<td>77</td>
<td>80</td>
</tr>
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</table>

Near confluent cells were incubated for 24 hours with various concentrations of the indicated compounds. The relative viable cell number was then determined by the MTT method (HSC-2, HSG, HL-60, HGF cells were assayed by trypan blue exclusion). Control CC50 of HSC-2, HSG and HGF cells were 1.376, 0.594 and 0.489, respectively. Each value represents the mean from 2 independent experiments which were performed in duplicate.

Table II. Effect of antioxidants and metals on the cytotoxic activity of compound 23.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxic activity of 23 against HSG cells (CC50: µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td>+ 4 mM NAC</td>
<td>25</td>
</tr>
<tr>
<td>+ 0.25 mM sodium ascorbate</td>
<td>28</td>
</tr>
<tr>
<td>+ 3000 unit/ml catalase</td>
<td>30</td>
</tr>
<tr>
<td>+ 0.2 mM CuCl2</td>
<td>35</td>
</tr>
<tr>
<td>+ 0.2 mM FeCl3</td>
<td>28</td>
</tr>
<tr>
<td>+ 0.2 mM CuCl2</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Near confluent HSG cells were incubated for 24 hours with various concentrations of compound 23 in the absence (control) or presence of the indicated concentrations of antioxidants or metals. The relative viable cell number was then determined by the MTT method to calculate the CC50 value. Control CC50 of HSG cells was 1.140. Each value represents the mean from 2 determinations.

Synthesis of 5-(2-hydroxy[benzoyl]-2-phenylimidazole (23). According to the general procedure C, 3-iodochromone (136 mg, 0.5 mmol) and benzamidine hydrochloride (313 mg, 2 mmol) were treated with K2CO3 for 0.5 hours to give 23 (128 mg, 97%).

Synthesis of 5-(2-hydroxy[benzoyl]-2-methylthioimidazole (24). According to the general procedure C, 3-iodochromone (136 mg, 0.5 mmol) and methylisonithioacetate (556 mg, 2 mmol) were treated with K2CO3 for 5 hours to give 24 (98 mg, 84%).

Cell culture. Human oral squamous cell carcinoma (HSC-2) cells human salivary gland tumor (HSG) cells and human gingival fibroblast (HGF) cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO2 atmosphere. Human promyelocytic leukemia HL-60 cells were cultured in RPMI1640 medium supplemented with 10% FBS (16).

Assay for cytotoxic activity. Near confluent HSC-2, HSG and HGF cells grown in 96-microwell plates (Falcon, flat bottom, treated polystyrene, Becton Dickenson) were incubated for 24 hours with various concentrations of samples. The cells were washed with phosphate-buffered saline and incubated for 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 with Labsystem Multiskan™ (Biochromatic) with Star/DOT Matrix Printer JIL-10. The 50% cytotoxic concentration (CC50) was determined from the dose-response curve (7).

Assay for redox potential. Compounds 7, 14, 18 or 23 (final 50 µg/mL) were added to 10 mL of DMEM medium supplemented with 10% FBS and mixed by constant stirring with a magnet stirrer. The redox potential was measured at the indicated time points thereafter with NO
monitor (Inter Medical Co., Ltd., Nagoya, Japan) and expressed as the
difference (AmV) from the initial value (17).

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 (16). DNA from
apoptotic HL-60 cells induced by UV irradiation (18) was run in parallel
as a positive control.

Results and Discussion

Among 24 compounds, compound 23 (5-(2-hydroxybenzoyl)-
2-phenylimidazole) showed the highest cytotoxic activity
against two human oral tumor cell lines (HSC-2, HSG)
(CC50=27 and 18 mg/mL, respectively) (Table I). Compounds 7 (2-(2-hydroxybenzoyl)benzimidazo[2,1-b] thiazole)
(CC50=52, 42 µg/mL), 14 (1,3-diethyl-5-(2-hydroxybenzoyl)-4-imidazoline-2-thione) (CC50=59, 35 µg/mL) and 18 (5-(2-
hydroxy-4-methoxybenzoyl)-3-methyl-2-methylimino-4-
thiazoline) (CC50=149, 13 µg/mL) showed slightly lower cyto-
toxic activity.

Tumor specificity. We found that normal fibroblasts (HGF cells)
were relatively resistant to compounds 7, 14 and 18
(tumor specific ratio (C/A)=3.5, 2.4 and 2.7, respectively, but
highly sensitive to compound 23 (C/A=0.89) (Table I). On
the other hand, human promyelocytic leukemia HL-60 cells
were very sensitive to all 4 compounds (C/B=11, 5.2, >40.0
and 5.8, respectively) (Table I).

Effect of antioxidants and metals. It was of interest to test the
possibility that compound 23 might induce cytotoxicity by its
prooxidant action. Table II shows that the cytotoxic activity of
compound 23 was not significantly affected by the optimum
concentrations of antioxidants, such as NAC (4 mM), sodium
ascorbate (0.25 mM) or catalase (3,000 unit/mL) (which
decomposes hydrogen peroxide). These data reduced the
possibility of prooxidant action of compound 23. Among 3
metals investigated, only CuCl2 significantly (5-fold)
enhanced the cytotoxic activity of compound 23, whereas
CoCl2 and FeCl3 were inactive (Table II).

Induction of DNA fragmentation. Agarose gel electrophoresis
showed that compound 23 dose-dependently induced DNA
fragmentation in HL-60 cells, but not in HSG cells (Figure 2).
However, there was a narrow range of optimal concentration,
and higher and lower concentrations failed to induce DNA
fragmentation. These data suggested that the response to
compound 23 might be different from cell to cell.

Among 24 compounds, we selected four cytotoxic
compounds 7, 14, 18 and 23. Compounds 7, 14, 18 selectively
killed the tumor cells. The most cytotoxic compound 23 did
not show such tumor-specific action, but induced DNA
fragmentation in HL-60 cells. We found that the cytotoxic
activity of compound 23 was not reduced by antioxidants, nor
by CoCl2. The lack of CoCl2 sensitivity can be explained by the
absence of the diol structure in the molecule (19). We
also found that CuCl2 enhanced the cytotoxic activity of
compound 23 by a yet unknown mechanism. We have recently
found that CuCl2 slightly enhanced the cytotoxic activity of 4-
chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5-
one and 2,3-dihydro-2-(2-oxopropyl)-2-phenyl-1-benzoepipin
(8). There might be common stimulation mechanisms
between these compounds. Further studies of the mechanism
of their cytotoxic action are under way in our laboratory.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of
Education, Science, Sports and Culture of Japan (No. 11671853).

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Received October 30, 2000
Accepted December 7, 2000