Tumor-specific Cytotoxicity and Apoptosis-inducing Activity of Berberines

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Abstract. Berberine iodide (IK-1) and acetoneberberine (IK-2) showed higher cytotoxicity against five human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4, NA, CA9-22) and one human promyelocytic leukemia (HL-60) cell lines, than against normal human oral tissue-derived cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF), producing a tumor specificity index of 4.0 and 3.6, respectively. IK-1 was more potent than IK-2 in inducing the production of apoptotic cells, internucleosomal DNA fragmentation, the activation of caspases-3, -8 and -9, and the increased expression of pro-apoptotic BAD protein, with a corresponding decrease in the expression of anti-apoptotic Bcl-2 protein in HL-60 cells. These compounds did not induce internucleosomal DNA fragmentation (only producing larger DNA fragment), nor increased the Bad protein expression in HSC-2 cells. The present study demonstrated the tumor-specific cytotoxicity and apoptosis-inducing activity of berberines, suggesting their possible antitumor potential.

Berberine is an alkaloid occurring in a number of plants such as Coptis japonica MAKINO and Phellodendron amurense RUPRECHT. It belongs to the group of isoquinoline derivatives and is a quaternary base. Berberine induced apoptotic cell death, characterized by reduced mitochondrial membrane potential, caspase-3 activation, DNA fragmentation and production of sub-G1-phase cells in the human tumor cell lines HL-60 (1, 2) and HeLa (3), and mouse tumor cell lines L1210 (3) and Balb/c 3T3 (4). A complex formation with DNA seems not to be associated with its apoptosis-inducing activity (2). On the contrary, berberine, at similar concentrations (10-1000 µM), displayed protective activity against tert-butyl hydroperoxide (t-BHP)-induced cytotoxicity (5), attenuated the apoptosis in ischemia and reperfusion-injured cardiac myocyte (6) and up-regulated the mdr1 gene product and reduced the antitumor action of Paclitaxel (7). Berberine also inhibited the apoptosis of murine thymocytes induced by dexamethasone, etoposide and camptothecin (8).

Cancer chemotherapy has traditionally been based on cytotoxic drugs that damage DNA directly, block its synthesis or interfere with the mechanisms of cell division. While treatment of certain malignancies with chemotherapy has been successful and encouraging, effectiveness has often been limited by side-effects on normal tissues and by the drug resistance of tumors. We have investigated hundreds of natural products for their tumor-specific cytotoxicity, and found that their apoptosis-inducing activity does not always correlate to tumor-specific cytotoxicity (9).

Berberine, a quaternary amine, shows the dependency of its hydrophobic interaction on pH and, therefore, possibly has low membrane permeability under physiological conditions. Thus, in this study, two berberine derivatives were prepared: berberine iodide (IK-1) and acetoneberberine (IK-2) (Figure 1), which are active irrespective of pH changes and are much more lipophilic than the original berberine (Figure 1). We investigated whether they induce tumor-specific cytotoxicity and apoptotic cell death, using six human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4, NA,
CA 9-22 and promyelocytic leukemia HL-60) and three normal human oral tissue-derived cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF). Internucleosomal DNA fragmentation, caspase activation and expression of apoptosis-related proteins were used as markers for apoptosis.

**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 Bioscience, Lenexa, KS, USA); 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); RNase A, proteinase K (Boehringer Mannheim, Germany); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan); DNA size marker (100 bp DNA Ladder, Bayou Biolabs, Harahan, LA, USA); DEVD-pNA (p-nitroanilide), IETD-pNA, LEHD-pNA (MBL, Nagoya, Japan); anti-Bcl-2 antibody, anti-Bad antibody (Santa Cruz Biotechnology, Delaware, Cam, USA); horseradish peroxidase (HRP)-linked anti-rabbit IgG, HRP-linked anti-mouse IgG (Amersham, Biosciences Corp., NJ, USA); anti-Actin antibody (Sigma).

**Preparation of berberine iodide (IK-1) and acetoneberberine (IK-2).** By the procedure reported in the literature (10), IK-1

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**Figure 1.** Structures of berberine iodide (IK-1) and acetoneberberine (IK-2) (upper panels) and the most stable (three-dimensional) structures (determined by CAChe Worksystem 4.9) viewed from two different directions (middle and lower panels).
(C₂₃H₁₈INO₄, MW 463.265, mp 260-262°C) and IK-2 (C₂₃H₂₃NO₅, MW 393.432, mp 264-267°C) (Figure 1) were prepared.

Cell culture. Normal human cells (HGF, HPC, HPLF) were obtained from human periodontal tissue after informed consent, according to the guidelines of the Meikai University Ethics Committee, Japan (No. 0206). Since normal cells have a limited lifespan (11), cells at 6-8 population doubling level (PDL) were used for the present study (12). HSC-2, HSC-4 and NA cells were supplied by Prof. Masao Nagumo, Showa University, Japan. HSC-3 and CA9-22 cells were supplied by Dr. Masakatsu Fukushima and Prof. Yoshihiro Ohmori, Meikai University, Japan, respectively. HL-60 cells were supplied by Prof. Kazuyasu 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 monolayer cultures at 37°C in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin.

Cytotoxic activity. The relative viable cell number of adherent cells was determined by the MTT method, while that of non-adherent cells (HL-60 cells) was determined by trypan blue dye exclusion. For the MTT assay, the cells were treated for 24 hours without (control) or with various concentrations of the 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 culture medium. For the trypan blue dye exclusion assay, the number of viable cells which did not incorporate the trypan blue dye was calculated by hemocytometer. 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 viable cells which did not incorporate the trypan blue dye was calculated by hemocytometer. From the dose-response curve, the 50% cytotoxic concentration (CC₅₀) was determined. Tumor-specific cytotoxicity (TS value) was determined by the following equation:

\[ \text{TS} = \frac{\text{CC}_{50}(\text{HGF}) + \text{CC}_{50}(\text{HPC}) + \text{CC}_{50}(\text{HPLF})}{\text{CC}_{50}(\text{HSC}-2) + \text{CC}_{50}(\text{HSC}-3) + \text{CC}_{50}(\text{HSC}-4) + \text{CC}_{50}(\text{NA}) + \text{CC}_{50}(\text{CA9}-22) + \text{CC}_{50}(\text{HL}-60)} \times (6/3) \]

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-lauroyl-sarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 hours at 50°C. After incubation, the lysis was mixed with 50 μL of NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and then with 100 μL of ethanol. After centrifugation for 4 minutes at 20,000 xg, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). A sample (10-20 μL) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) (13). DNA from apoptotic HL-60 cells induced by actinomycin D (1 μg/mL) was used for calibration. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

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 xg, at 4°C for 5 minutes, the supernatant was collected. To the 50-μL sample (equivalent to 0.1 mg protein), 55 μL of 2 x reaction buffer (MBL) containing substrate for caspase-3 (DEVD-pNA), 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 by the cleavage of substrates was measured by microplate reader, according to the manufacturer's instruction (MBL) (13).

Western blotting. The cell pellets were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice water, and then incubated for 50 minutes at 4°C with RT-5 ROTATOR (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 minutes at 4°C to remove the insoluble materials and collect the supernatant. The protein concentrations of the supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from the cell lysates (10 μg) was mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercaptopoethanol), boiled for 10 minutes, applied to the SDS-7% polyacrylamid gel electrophoresis and then transferred to polyvinilidene difluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk in PBS(–) plus 0.05% Tween 20 for 90 minutes and incubated with anti-Bcl-2 antibody (1:1,000), anti-Bad antibody (1:1,000) or anti-Actin antibody (1:1,000) for 90 minutes at room temperature. After incubation for 60 minutes at room temperature with HRP-conjugated anti-lgG (1:2,000), the immunoblots were detected by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) (13).

Calculation. The optimization of the structure was done by a semi-empirical molecular-orbital method (MOPAC, PM5, non-COSMO), using CAChe Worksystem 4.9 (Fujitsu Co. Ltd., Tokyo, Japan). The octanol-water distribution coefficient (log P) was calculated from the heat of formation, using CAChe Worksystem 4.9 (14).

Results

Tumor-specific cytotoxic action. Berberine iodide (IK-1) has a planar structure, whereas acetoneberberine (IK-2) has a protrusion of the attached CH₂COCH₃ (Figure 1). These compounds showed higher cytotoxicity against five human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4, NA, CA9-22) and one human promyelocytic leukemia (HL-60) cell lines than against normal human oral tissue-derived cells (HGF, HPC, HPLF), producing a tumor specificity index (TS) of 4.0 and 3.6, respectively (Table I). HL-60 cells were most sensitive to both IK-1 and IK-2 (CC₅₀=18-22 μM), followed by HSC-2, HSC-3, HSC-4 and NA cells (CC₅₀=47-88 μM). CA 9-22 cells were the most resistant to these compounds (CC₅₀=132-136 μM) (Table I).

Apoptosis induction. When HL-60 cells were incubated for 6 hours with IK-1 or IK-2, apoptotic cells began to appear...
above 6.25 μM, and reached a maximum level at 100 μM (Figure 2). The effect of IK-1 slightly exceeded that of IK-2.

Both IK-1 and IK-2 induced internucleosomal DNA fragmentation in HL-60 cells, whereas they induced the production of a large DNA fragment (indicated by arrows) in HSC-2 cells (Figure 3).

Both compounds induced activation of caspases-3, -8 and -9 in HL-60 cells (upper panel in Figure 4). Their stimulation activity appeared above 10-20 μM and reached a maximum level at 80 μM, a comparable level attained by actinomycin D, a positive control. The stimulation effect of IK-1 slightly exceeded that of IK-2. Both IK-1 and IK-2 also activated all three caspases in HSC-2 to a level slightly exceeding that attained by actinomycin D (lower panel in Figure 4).

When HL-60 cells were treated with lower concentrations (10 or 20 μM) of IK-1, the expression of Bad protein was enhanced and that of Bcl-2 declined (left panel in Figure 5). However, such changes in Bad and Bcl-2 disappeared at higher concentrations. On the other hand, IK-2 did not show a significant effect on Bad and Bcl-2 expressions at any concentrations (10-80 μM) (right panel in Figure 5).

Discussion

The present study demonstrated that both IK-1 and IK-2 induced apoptosis in HL-60 cells, in consistence with previous papers (1, 2). The apoptosis-inducing activity of berberines between our results (6-100 μM) and theirs (75 μM) was similar. The relatively higher cytotoxicity of these compounds may be due to their favorable hydrophobicity [log P=2.256 (IK-1) and 2.386 (IK-2)], since the cytotoxicity of Eugenol-related compounds (15), vitamin K derivatives (16) and flavonoids (17) became maximum when their log P value reached around 3. It was found that the tumor-specific cytotoxicity and apoptosis-inducing activity of IK-1 were slightly higher than those of IK-2, based on the number of apoptotic cells, the extent of caspase activation and Bad protein expression. This may be due to the difference of solubility between IK-1 (with iodide) and IK-2 (with CH₃COCH₃). There is an alternate possibility that acetone may be released by IK-2 degradation and affect the cellular metabolism.

We found that IK-1, only at the selected concentration (20 μM), enhanced the expression of pro-apoptotic Bad protein in HL-60 cells, whereas it reduced the expression of the anti-apoptotic Bcl-2 protein. It remains to be investigated whether IK-1 changes the subcellular localization and phosphorylation status of Bad, since the cleavage of 14-3-3 protein (anti-apoptotic factor) by caspase-3 reduced the binding affinity to Bad and released Bad to translocate to the mitochondrial outer membrane (18), and rotenone-induced apoptosis is coupled with dephosphorylation of Bad without changing the amount of

<table>
<thead>
<tr>
<th>Normal cells</th>
<th>Tumor cell lines</th>
</tr>
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<tbody>
<tr>
<td>HGF</td>
<td>HPC</td>
</tr>
<tr>
<td>I</td>
<td>&gt;400</td>
</tr>
<tr>
<td>II</td>
<td>293</td>
</tr>
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Near confluent cells were incubated for 24 hours with various concentrations of berberines, and the relative viable cell number was determined by MTT or trypan blue dye exclusion. The 50% cytotoxic concentration was determined from the dose-response curve.
Bad (19). Contrary to our findings, it has been reported that berberine inhibited COX-2 and Mcl-1 expression and Akt phosphorylation, without affecting the Bcl-2 expression in oral cancer KB cells (20). We also observed that IK-2 failed to enhance the Bad protein expression and reduce the Bcl-2 expression in HL-60 cells (Figure 5), and that both IK-1 and IK-2 failed to modify the expressions of Bad and Bcl-2 in human oral squamous cell carcinoma HSC-2 cells (unpublished data). This suggests that berberines induce apoptosis markers, depending on which target cells are analyzed. Oral cancer cells may be resistant to apoptosis induction by berberines.

Berberine has been reported to down-regulate nucleophosmin/B23 and telomerase activity in HL-60 cells (21), and inhibit arylamine N-acetyltransferase (NAT) activity, mRNA expression and protein level in murine L1210 cells (22). The biological significance of this phenomenonon is still unclear and may differ from cell to cell.

We found that two of the berberines, especially IK-1, showed both tumor-specific cytotoxicity and apoptosis-inducing activity, although the TS value was not as high as the anthracyclins (9). This suggests the possible antitumor potential of IK-1 in a future clinical application.

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References

Figure 4. Activation of caspases by berberines in HL-60 and HSC-2 cells. HL-60 (upper panel) and HSC-2 (lower panel) cells were incubated for 4 hours without (control) or with the indicated concentrations of IK-1 or IK-2, or 1 μg/mL actinomycin D and the activity of caspases-3 (black bar), -8 (gray bar) or -9 (open bar) was determined by substrate cleavage assay, and expressed as % of control.

Figure 5. Effect of berberines on the expression of Bad and Bcl-2 proteins. HL-60 cells were incubated for 4 hours with the indicated concentrations of IK-1 or IK-2, or 1 μg/ml actinomycin D, and the cell lysates were subjected to Western blot analysis with specific antibodies against Bad, Bcl-2 or Actin. The extent of the expression of Bad and Bcl-2 proteins was plotted as the ratio to that of Actin.


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