

Induction of Non-apoptotic Cell Death by Morphinone in Human Promyelocytic Leukemia HL-60 Cells

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Abstract. As previously suggested, codeinone (oxidation product of codeine) induces non-apoptotic cell death, characterized by marginal caspase activation and the lack of DNA fragmentation in HL-60 human promyelocytic leukemia cells, which was inhibited by N-acetyl-L-cysteine. Whether, morphinone, an oxidative metabolite of morphine, also induced a similar type of cell death in HL-60 cells was investigated. Morphinone showed slightly higher cytotoxic activity against human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4, NA, Ca9-22, promyelocytic leukemia HL-60, cervical carcinoma HeLa) than against normal oral human cells (gingival fibroblast HGF, pulp cells HPC, periodontal ligament fibroblast HPLF). Morphinone also induced an almost undetectable level of internucleosomal DNA fragmentation in the HL-60 cells. Morphinone did not activate caspase-8 or -9 in these cells. Morphinone dose-dependently activated caspase-3 in both HL-60 and HSC-2 cell lines, but to a much lesser extent than actinomycin D. Electron microscopy demonstrated that morphinone induced mitochondrial shrinkage, vacuolization and production of autophagosome and the loss of cell surface microvilli, without destruction of cell surface and nuclear membranes in the HL-60 cells. The autophagy inhibitor 3-methyladenine (0.3~10 mM) slightly inhibited the morphinone-induced cytotoxicity, when corrected for its own cytotoxicity. These data suggest that morphinone induces non-apoptotic cell death in HL-60 cells.

Morphinone is an oxidation product of morphine, a narcotic analgesic used to relieve pain (1). Opioids are used to relieve pain in cancer treatment according to the WHO guidelines (2). In line with Ventafridda *et al.*, pain appears in more than 50% of cancer patients and the administration of opioids manifested significantly higher therapeutic effects on the cancer pain, compared with non-narcotics (3, 4). Although opioids are used in patients under clinical treatments, the anticancer action of opioids is not well investigated. Both opioids and anticancer drugs have been simultaneously given to relieve the pain of cancer, but the underlying mechanism of this combined antitumor effect is unclear. While, morphine was proposed as the third step on the analgesic ladder by the WHO cancer treatment guidelines, its apoptosis-inducing ability is not understood well. Recently, codeinone, an oxidation product of codeine, was reported to show higher cytotoxic activity against various cancer cell lines (5-7), compared with codeine, suggesting its tumor-specific cytotoxicity. There are at least three types of cell death – apoptosis, autophagy and necrosis (8). We have recently reported that codeinone induced non-apoptotic cell death (characterized by lack of DNA fragmentation and caspase activation) in HL-60, promyelocytic leukemia cell lines (9). In the present study, we investigated whether morphinone (Figure 1), having structural similarity with codeinone, can induce a similar type of cell death in HL-60 cells.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI1640, Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); dimethylsulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan). Morphinone was synthesized as described previously (10).

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Key Words: Morphinone, α,β -unsaturated ketones, anticancer drugs, tumor-specificity, non-apoptotic cell death, mitochondria.

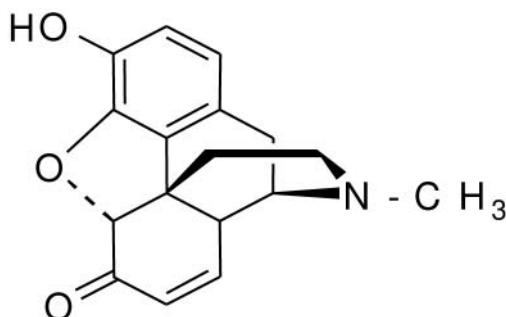


Figure 1. Structure of morphinone.

Cell culture. HL-60 cells were obtained from Prof. K. Nakaya, Showa University, Japan, and cultured in RPMI 1640 medium supplemented with 10% FBS. Human oral squamous cell carcinoma cells (HSC-2, HSC-3, HSC-4, NA, Ca9-22) were obtained from Prof. M. Nagumo, Showa University, Japan. Human cervical carcinoma HeLa cells were obtained from Dr. K. Sugiyama, Meikai University, Japan. These adherent cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. Normal human oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) were prepared from the periodontal tissues, following the guidelines of the Meikai University Ethics Committee (No. 0206), after obtaining the informed consent from the patients. Since normal oral cells have limited life-span and cease proliferation at the 20 population doubling level (PDL) (11), these cells were used at the 5-9 PDL in the present study.

Assay for cytotoxic activity. HL-60 cells were inoculated at 1.0×10^6 cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 h, the medium was removed with an aspirator and replaced with 0.1 mL of fresh medium containing the indicated concentrations of morphinone. Cells were incubated for another 24 h and the relative viable cell number was then determined by the MTT method. In brief, cells were washed once with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(-)] and replaced with fresh culture medium containing 0.2 mg/mL MTT. After 4-h incubation, the cells were lysed with 0.1 mL of DMSO and the absorbance at 540 nm of the cell lysate was determined, using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The absorbance at 540 nm of control cells was usually in the range of 0.40 to 0.90. From the dose-response curve, the 50% cytotoxic concentration (CC_{50}) of morphinone was determined. The tumor-specificity index (TS) was defined by the following equation: $\text{TS} = \{\frac{[\text{CC}_{50}(\text{HGF}) + \text{CC}_{50}(\text{HPC}) + \text{CC}_{50}(\text{HPLF})]}{[\text{CC}_{50}(\text{HSC-2}) + \text{CC}_{50}(\text{HSC-3}) + \text{CC}_{50}(\text{HSC-4}) + \text{CC}_{50}(\text{NA}) + \text{CC}_{50}(\text{Ca9-22}) + \text{CC}_{50}(\text{HeLa}) + \text{CC}_{50}(\text{HL-60})]}\} \times (7/3)$.

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-lauroylsarcosinate]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/mL proteinase K for 1-2 h at 50°C and were then mixed with 50 μL NaI solution [7.6 M NaI, 20 mM EDTA-2Na,

Table I. Tumor-specific cytotoxicity of morphinone.

Cells	CC_{50} (μM) of morphinone
Normal cells	
HGF	90 \pm 79
HPC	172 \pm 153
HPLF	79 \pm 42
Other tumor cell lines	
HSC-2	44 \pm 3
HSC-3	52 \pm 11
HSC-4	38 \pm 19
NA	47 \pm 17
Ca9-22	24 \pm 12
HL-60	25 \pm 11
HeLa	31 \pm 20

Each value represents mean \pm S.D. from 4 independent experiments.

40 mM Tris-HCl, pH 8.0], and 100 μL of ethanol. After centrifugation for 20 min 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 8.0). The 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). DNA molecular weight marker (Takara) and the DNA from apoptotic HL-60 cells induced by 1 $\mu\text{g}/\text{ml}$ actinomycin D were used for calibration (12). The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay for caspase activation. Cells were washed with PBS(-) and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 min on ice and centrifugation for 5 min at 10,000 xg, the supernatant was collected. Lysate (50 μL , equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-pNA (*p*-nitroanilide)), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA). After incubation for 2 h at 37°C, the absorbance at 405 nm of the liberated chromophore pNA was measured by microplate reader (13, 14).

Electron microscopy. The control and morphinone-treated cells were harvested by trypsin-EDTA and were pelleted by centrifugation at 1,000 rpm for 5 min. The cells were fixed for 1 h with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C, postfixed for 1 h with 1% osmium tetroxide-0.1 M cacodylate buffer (pH 7.4) at 4°C, dehydrated and then embedded in Araldite 502 (CIBA-GEIGY Swiss; NISSHIN EN Co., Ltd., Tokyo, Japan). Fine sections were stained with uranyl acetate and lead citrate, and were then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 120 KV (15). The mitochondrial area (S) was calculated by the following equation: $S = \pi ab/4$, where a and b long are shorter and longer diameters of mitochondria, respectively, measured on the enlarged photograph (see Figure 5).

Results

Tumor-specificity. Morphinone (Figure 1) showed higher cytotoxicity against human tumor cell lines including five oral

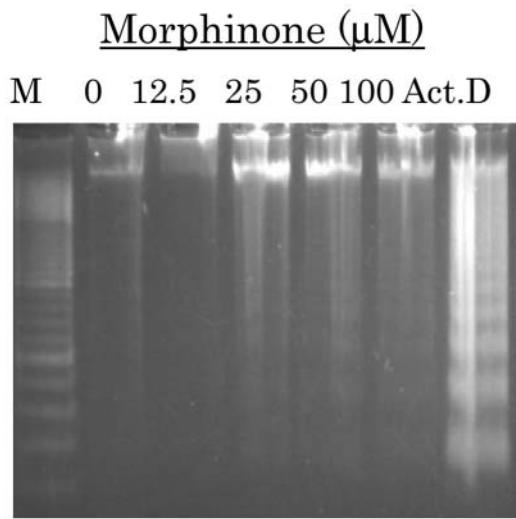


Figure 2. Morphinone does not induce internucleosomal DNA fragmentation in HL-60 cells. HL-60 cells were incubated for 6 h with the indicated concentrations of morphinone or 1 μ g/ml actinomycin D (Act. D). DNA was then extracted and applied to agarose gel electrophoresis. Data are the representation of three independent experiments. M: DNA marker.

squamous cell carcinomas [HSC-2 ($CC_{50}=44 \mu\text{M}$), HSC-3 ($CC_{50}=52 \mu\text{M}$), HSC-4 ($CC_{50}=38 \mu\text{M}$), NA ($CC_{50}=47 \mu\text{M}$), Ca9-22 ($CC_{50}=24 \mu\text{M}$)], one promyelocytic leukemia HL-60 [$CC_{50}=25 \mu\text{M}$] and cervical carcinoma HeLa ($CC_{50}=31 \mu\text{M}$) than three human oral normal cells [gingival fibroblast HGF ($CC_{50}=90 \mu\text{M}$), pulp cells HPC ($CC_{50}=172 \mu\text{M}$), periodontal ligament fibroblast HPLF ($CC_{50}=79 \mu\text{M}$)] (Table I), yielding a tumor-specificity index of 3.0.

Induction of non-apoptotic cell death. Morphinone only marginally induced internucleosomal DNA fragmentation in HL-60 cells, in contrast to actinomycin D (Figure 2). Morphinone (0~100 μM) activated caspase-3 dose-dependently, but to a much lesser extent than that by actinomycin D. Morphinone failed to activate caspase-8, or -9 (Figure 3).

The electron microscopy (EM) study revealed that morphinone induced the loss of microvilli in the cell surface membrane, but the membrane integrity and appearance of nucleoli were maintained (Figure 4C-4E). Morphinone failed to produce apoptotic bodies, in contrast to actinomycin D (Figure 4F). Morphinone dose-dependently reduced the mitochondrial size; at 80 μM the mitochondrial area was reduced to 1/4 of control lines, but was not significantly reduced by actinomycin D (Table II). Morphinone also induced vacuolization and the production of autophagosome in which organelles were engulfed (Figure 5), suggesting the occurrence of autophagy. To test this possibility, the effect of 3-methyladenine, an inhibitor of

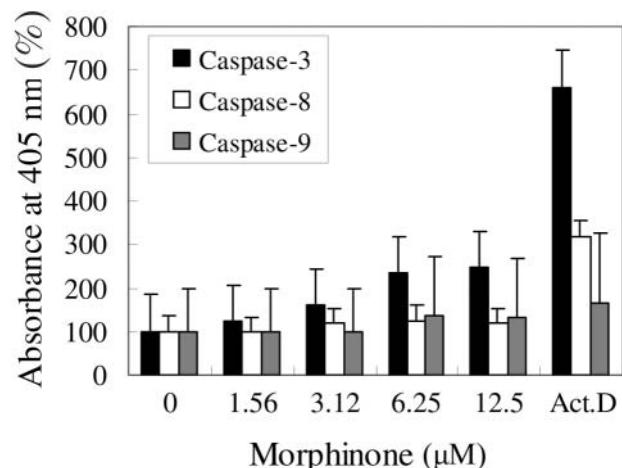


Figure 3. Morphinone did not activate caspase-3, -8 or -9 in HL-60 cells. HL-60 cells were incubated for 4 h with the indicated concentrations of morphinone or 1 $\mu\text{g}/\text{ml}$ actinomycin D (Act. D). Caspase activity was then assayed and expressed as % of control. The absorbances at 405 nm of the cleaved product by caspase -3, -8, -9 in control cells were 0.051, 0.042 and 0.031, respectively.

Table II. Induction of mitochondrial shrinkage by morphinone in HL-60 cells.

Morphinone (μM)	Mitochondrial area (μm^2)
0	0.51 \pm 0.17 (n=55)
10	0.37 \pm 0.06 (n=67)
20	0.27 \pm 0.07 (n=66)
40	0.17 \pm 0.03 (n=113)
80	0.13 \pm 0.05 (n=129)
Act.D (1 mg/mL)	0.42 \pm 0.03 (n=49)

Each value represents mean \pm S.D. from the indicated number of samples.

Act. D: actinomycin D.

autophagy, was tested on morphinone-induced cell death (Figure 6). The HL-60 cells were highly sensitive to 3-methyladenine and their viability was reduced by 45% at 10 mM (Figure 6A), a concentration previously used to inhibit autophagy (8, 16, 17). When corrected for the cytotoxicity of 3-methyladenine, morphinone-induced cell death was found to be slightly inhibited by 3-methyladenine (Figure 6B).

Discussion

The results of the present study demonstrate that morphinone induced non-apoptotic cell death, based upon the following evidence: (a) morphinone induced a much lower level of internucleosomal DNA fragmentation, caspase activation and

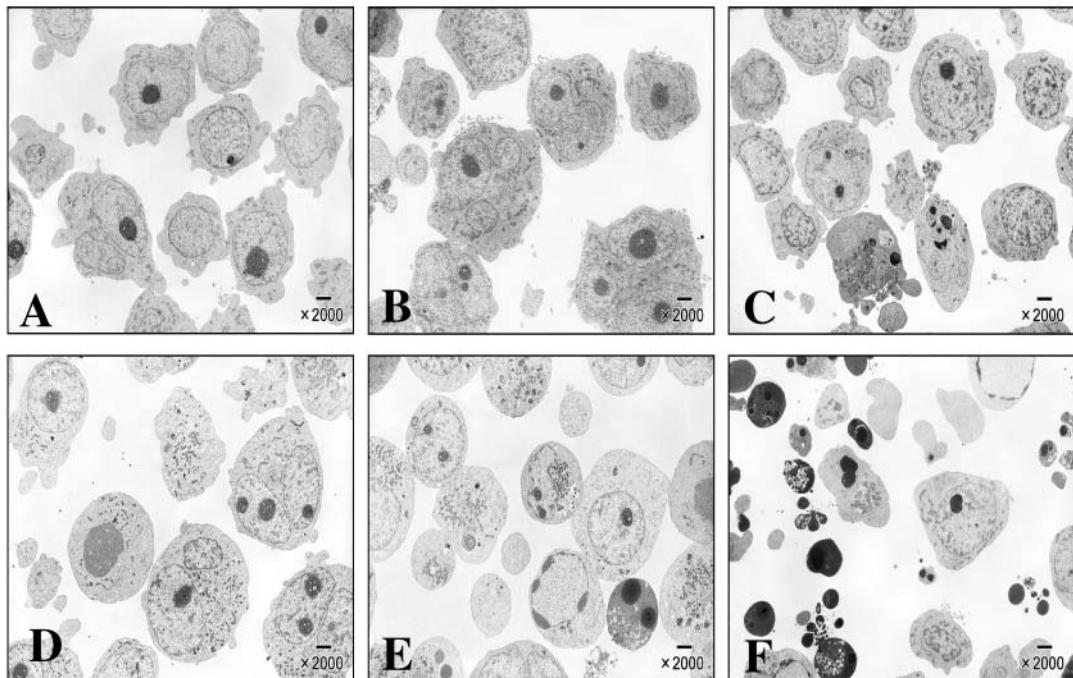


Figure 4. Morphological changes induced by morphinone in HL-60 cells. HL-60 cells were incubated for 6 h with 0 (A), 10 (B), 20 (C), 40 (D) or 80 μM morphinone (E), or 1 $\mu\text{g}/\text{ml}$ actinomycin D (F) and were then processed for the electron microscopy. Bar: 1 μm .

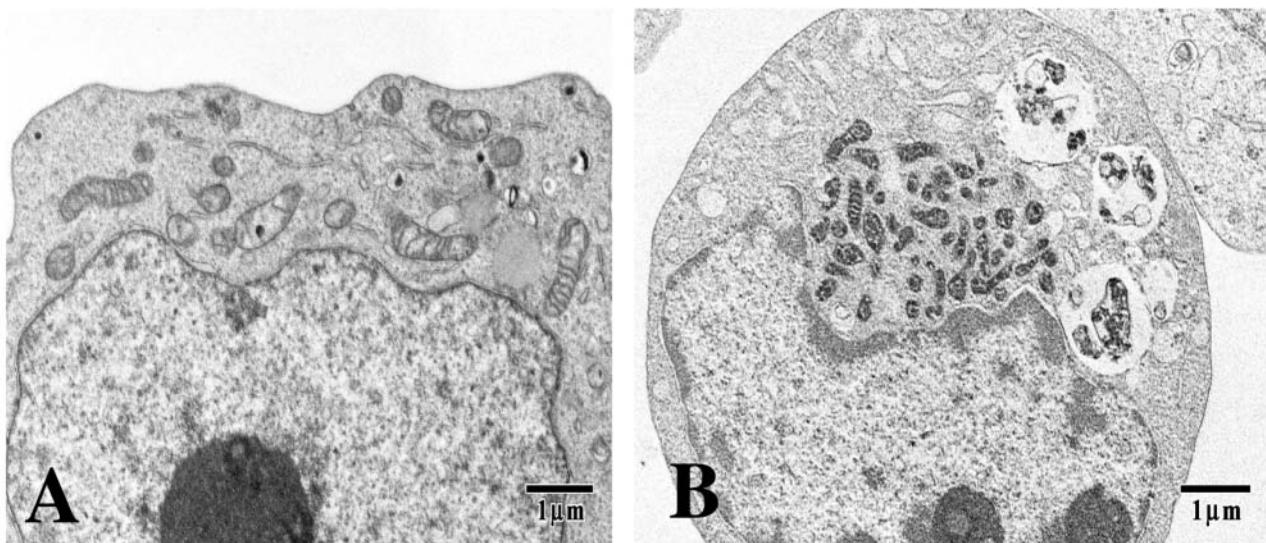


Figure 5. Appearance of autophagosome by morphinone. HL-60 cells were incubated for 6 h with 0 (A) or 80 μM morphinone (B) and were processed for electron microscopy. Bar: 1 μm .

apoptotic body production, compared with actinomycin D, a positive control and (b) morphinone induced mitochondrial shrinkage and vacuolization, in contrast to actinomycin D. We found that morphinone induced the formation of autophagosomes, in which engulfed organelles were

accumulated for auto-degradation. This suggests that morphinone, at slightly higher concentration (approximately 3.2-times higher concentration of CC_{50}), induces autophagy, a second type of programmed cell death. Mitochondrial shrinkage may lead to the functional loss of this organelle and

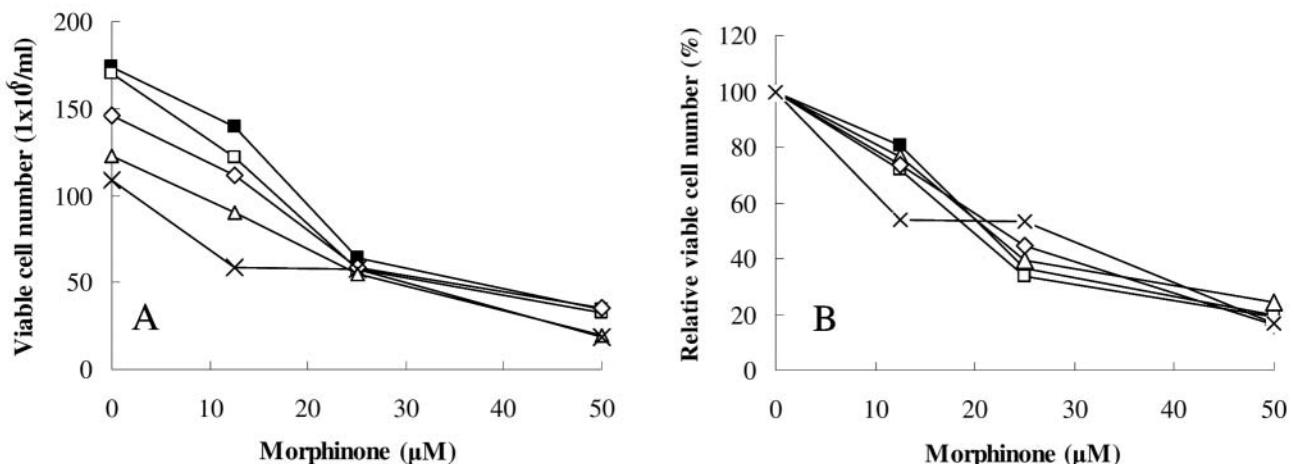


Figure 6. Effect of 3-methyladenine on morphinone-induced cytotoxicity. (A) HL-60 cells were incubated for 24 h with the indicated concentrations of morphinone in the presence of 0 (■), 0.3 (□), 1 (◇), 3 (△) or 10 mM (×) 3-methyladenine and the viable cell number was determined by trypan blue dye exclusion. Each value represents mean from three determinations. S.D. < 10%. (B) Data were corrected for 3-methyladenine cytotoxicity.

ATP depletion. However, 3-methyladenine, an autophagy inhibitor, only marginally inhibited the morphinone-induced cell death in HL-60 cells. The lower sensitivity to this inhibitor may be due to the incomplete induction of autophagy or induction of other unidentified types of cell death in the HL-60 cells by morphinone.

The type of cell death may be determined not only by the cell types, but also by the types of compounds administered. We recently reported that codeinone and other seven simple cyclic α,β -unsaturated ketones (2-cyclohexen-1-one, 2-cyclopenten-1-one, 4,4-dimethyl-2-cyclopenten-1-one, 2-cyclohepten-1-one, α -methylene- γ -butyrolactone, 5,6-dihydro-2H-pyran-2-one, methyl 2-oxo-2H-pyran-3-carboxylate) activated caspases-3, -8 and -9 very weakly (13). When these compounds were treated with *N*-acetyl-L-cysteine, their β -carbon was blocked by SH group of NAC via Michael reaction, losing their cytotoxicity. This suggests that their cytotoxic action may be produced by blocking SH-enzymes. It remains to be clarified whether all α,β -unsaturated ketones induce non-apoptotic cell death or not. Recently, malignant glioma cells were shown to be susceptible to undergo autophagy (16). Whether glioma and other tumor cell lines undergo autophagy by morphinone should be investigated.

The present data demonstrate that morphinone displays tumor-specific cytotoxicity, in addition to its analgesic action. Further studies are required for the consideration of morphinone as a candidate for the treatment of cancer.

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