

## Effect of Anticancer Agents on Codeinone-induced Apoptosis in Human Cancer Cell Lines

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**Abstract.** *The possible apoptosis-inducing activity of codeinone, an oxidative metabolite of codeine, without or with anticancer drugs, was investigated. Codeinone induced internucleosomal DNA fragmentation in human promyelocytic leukemia cells (HL-60), but not in human squamous cell carcinoma cells (HSC-2). Codeinone dose-dependently activated caspase-3 in both of these cells, but to a much lesser extent than that attained by actinomycin D. This property of codeinone was similar to what we have found previously in  $\alpha,\beta$ -unsaturated ketones. Codeinone did not activate caspase-8 or caspase-9 in these cells. The cytotoxic activity of codeinone against HSC-2 cells was inhibited by N-acetyl-L-cysteine, but somewhat additively stimulated by sodium ascorbate, epigallocatechin gallate, hydrogen peroxide, sodium fluoride, 5-fluorouridine, cisplatin, doxorubicin and methotrexate. These data suggest that codeinone has possible antitumor potential, in addition to its action as a narcotic analgesic, even though it induces incomplete apoptosis-associated characteristics.*

Codeinone (Figure 1) is an oxidation product of codeine, a narcotic analgesic used to relieve pain (1). In both humans and animals, the major metabolites of codeine are codeine 6-glucuronide, morphine and norcodeine (2). However, in the presence of nicotinamide-adenine dinucleotide (NAD), the 9000 xg supernatant of guinea pig liver homogenate can transform codeine into codeinone (3).

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Opioids have been given to patients with cancer pain, according to the WHO ladder against pain (4). According to reports of Ventafridda *et al.*, pain occurs in more than 50% of cancer patients, and the administration of opioids manifests significantly higher therapeutic effects against cancer pain, compared with non-narcotics (5, 6). Although opioids have been used for patients in clinical situations, the anticancer action of opioids has not been well investigated. Both opioids and anticancer drugs have been simultaneously given for pain in cancer patients. However, there has been no investigation of the antitumor effect of simultaneously administered drugs. Furthermore, codeine, an opioid used as the second step on the analgesic ladder proposed by the WHO cancer treatment guidelines, has not yet been investigated for its apoptosis-inducing ability.

We have recently found that codeinone had high cytotoxic activity against various cancer cell lines (7-9), as compared with codeine, suggesting its applicability for the treatment of cancer. In the present study, whether codeinone can induce apoptosis (characterized by internucleosomal DNA fragmentation, caspase activation) in two human tumor cell lines, promyelocytic leukemia (HL-60) and squamous cell carcinoma (HSC-2) was investigated. In addition, whether other popular anticancer drugs can reinforce its cytotoxic action was also examined.

### 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), etoposide, 3-[4,5-dimethylthiazol-2yl]-2, 5-diphenylterazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); dimethylsulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); sodium fluoride (NaF), methotrexate (amethoptrin, Nacalai Tesque, Inc., Kyoto, Japan); doxorubicin, 5-fluorouridine (5-FU) (Kyowa, Tokyo, Japan); cisplatin (Briplatin injection, Bristle Pharmaceutical Co., Tokyo, Japan); N-acetyl-L-

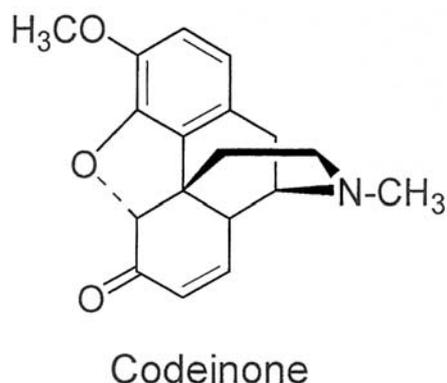


Figure 1. The structure of codeinone.

cysteine (NAC). Codeinone was synthesized as described previously (7).

**Cell culture.** HL-60 cells (RIKEN) were cultured at 37°C in RPMI 1640 supplemented with 10% heat-inactivated FBS. HSC-2 cells (kindly supplied by Prof. Nagumo, Showa University, Japan) were cultured in DMEM supplemented with 10% FBS under a humidified 5% CO<sub>2</sub> atmosphere.

**Assay for cytotoxic activity.** HSC-2 cells were inoculated at 12x10<sup>3</sup> cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 h, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing various concentrations of the test compounds. The cells were incubated for another 24 h, and the relative viable cell number was then determined by the MTT method. In brief, the cells were washed once with phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup> [PBS(-)], and replaced with fresh culture medium containing 0.2 mg/mL MTT. After incubation for 4 h, 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.

**Assay for DNA fragmentation.** Cells were lysed with 50 µL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate]. 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 then mixed with 50 µL NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and 100 µL of ethanol. After centrifugation for 20 min at 20,000 x g, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 3-5). 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). The DNA molecular marker (Takara) and the DNA from apoptotic HL-60 cells induced by ultraviolet (UV) irradiation were used for calibration (10). 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

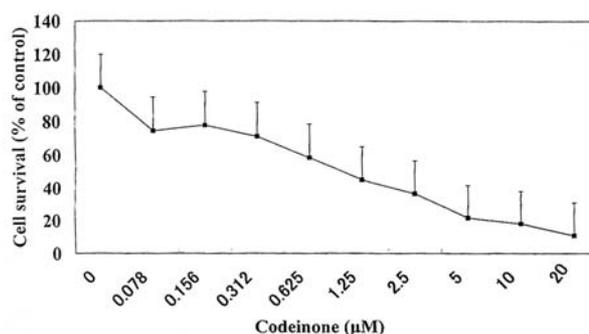


Figure 2. Effect of codeinone on the growth of HSC-2 cells. Near confluent HSC-2 cells were incubated for 24 h with the indicated concentrations of codeinone, and then assayed for the viable cell number by the MTT method (expressed as % of control). Each value represents mean  $\pm$  S.D. of three determinations.

min on ice and centrifugation for 5 min at 10,000 x g, the supernatant was collected. The lysate (50 µL, equivalent to 200 µg protein) was mixed with 50 µL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for 2 h at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by a microplate reader (11-13).

## Results

**Induction of apoptosis by codeinone.** Codeinone dose-dependently reduced the viable cell number of HSC-2 cells (0 ~ 20 µM). At 10-20 µM of codeinone, the viability of the cells was reduced to below 20% (Figure 2). Codeinone failed to show growth promotion at lower concentrations (so-called "hormesis") in HSC-2 cells (Figure 2). Codeinone induced internucleosomal DNA fragmentation in HL-60 cells, but not in HSC-2 cells (Figure 3). Furthermore, codeinone (0 ~ 10 µM) activated caspase-3, but not caspase-8, or caspase-9 in both HL-60 and HSC-2 cells (Figure 3). The extent of caspase-3 activation by codeinone was much lower than that attained by actinomycin (1 µg/mL), positive control (Figure 3).

**Combination effect of codeinone and antitumor agent.** The cytotoxic activity of codeinone was dose-dependently inhibited by *N*-acetyl-L-cysteine (NAC) (Figure 4A), confirming our previous finding (14). The inhibitory effect of NAC was observed above 0.25 mM, and reached maximum level at 2 mM. It can be seen in Figure 4 that sodium ascorbate (VC) (B), epigallocatechin gallate (EGCG) (C), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (D), NaF (E), 5-FU (F), cisplatin (G), doxorubicin (H) and methotrexate (I) dose-dependently reduced the viable cell number of HSC-2 cells. Non-cytotoxic concentrations of codeinone did not enhance the cytotoxic action of these compounds. Likewise, non-cytotoxic concentrations of these compounds did not enhance the

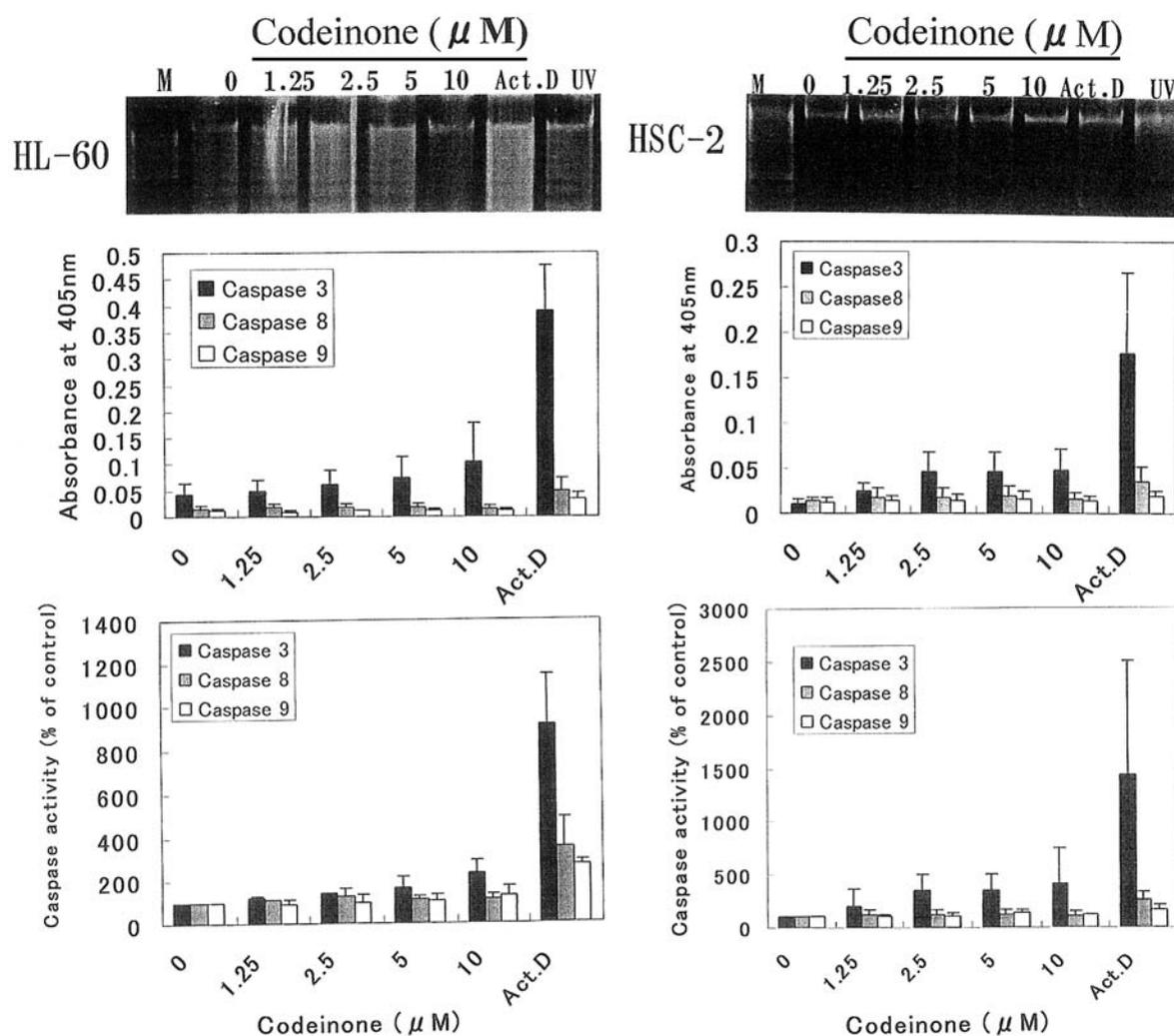


Figure 3. Effect of codeinone on apoptosis. HL-60 or HSC-2 cells were incubated for 4 or 6 h with the indicated concentrations of codeinone, and assayed for internucleosomal DNA fragmentation by agarose gel electrophoresis (upper panels) or caspase activation by substrate cleavage method (middle panels) expressed as absorbance at 540/620 nm of pNA (C). M, marker DNA. Act. D, 1  $\mu\text{g/mL}$  actinomycin D. UV, DNA from apoptotic HL-60 cells induced by UV irradiation (10). Caspase activity was also expressed as % of control (without drug) (lower panels). Each value represents mean  $\pm$  S.D. from 4 different experiments.

cytotoxic activity of codeinone. The combination of slightly cytotoxic concentrations of these compounds (Figure 4A-I) and codeinone somewhat additively stimulated their cytotoxic activity (Figure 4).

## Discussion

The present study demonstrated that codeinone dose-dependently reduced the viable cell number of two tumor cell lines (HL-60, HSC-2), without inducing hormesis (growth promoting action at lower concentrations). We also found that NaF, an inhibitor of glycolysis, did not induce hormesis at lower concentrations (15). This

suggests that not all cytotoxic agents induce hormesis (16). The present study also demonstrated that codeinone induced internucleosomal DNA fragmentation in HL-60 cells, but not in HSC-2 cells. It was unexpected that codeinone activated caspase-3, only at concentrations higher than that required for DNA fragmentation, whereas it did not activate caspase-8 or caspase-9. Furthermore, the extent of caspase-3 activation induced by codeinone was much lower than that attained by actinomycin D. These data suggest that codeinone may induce necrosis (characterized by cell swelling) or autophagy (characterized by vacuolization and expression of ATG 7 and beclin 1) in non-apoptotic cells (17-19).

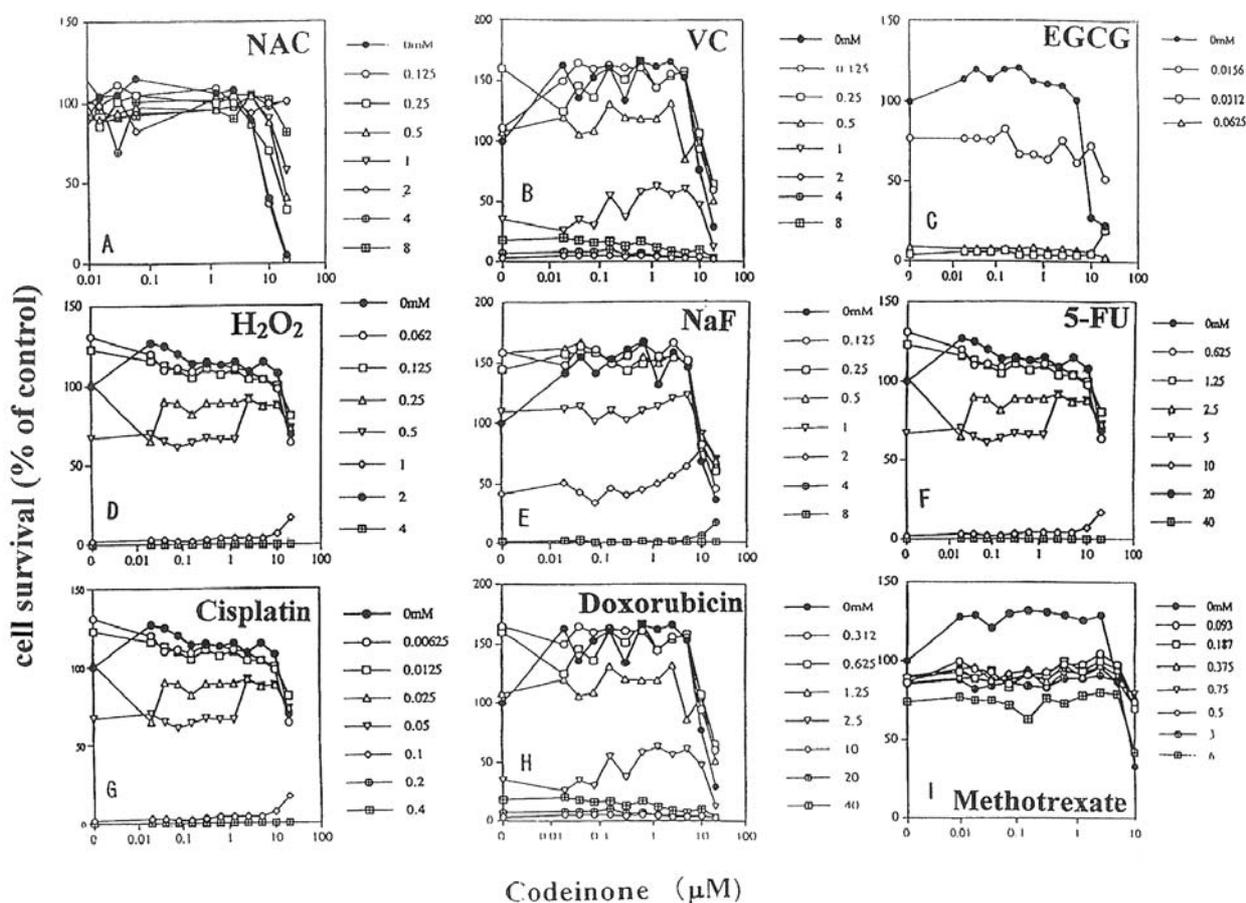


Figure 4. Combination effect of codeinone and antitumor agents on the growth of HSC-2 cells. Near confluent HSC-2 cells were incubated for 24 h with the indicated concentrations of codeinone, together with NAC (A), sodium ascorbate (VC) (B), EGCG (C), H<sub>2</sub>O<sub>2</sub> (D), NaF (E), 5-FU (F), cisplatin (G), doxorubicin (H), or methotrexate (I). The relative viable cell number was then determined by the MTT method and expressed as % of control (without compound). Each point represents mean from three determinations.

Codeinone has an  $\alpha,\beta$ -unsaturated ketone group. We have previously reported that  $\alpha,\beta$ -unsaturated ketones, such as 2-cyclohexene-1-one, 2-cyclopentene-1-one, 4,4-dimethyl-2-cyclopentene-1-one, 2-cycloheptene-1-one,  $\alpha$ -methylene- $\gamma$ -butyrolactone, 5,6-dihydro-2H-pyran-2-one and methyl 2-oxo-2H-pyran-3-carboxylate activated only caspase-3, but not caspase-8 or -9 (11). Further study is required to test the possibility that specific activation of caspase-3 is a general phenomenon observed for  $\alpha,\beta$ -unsaturated ketone-induced cytotoxicity.

The present study also demonstrated that the cytotoxic action of codeinone was not greatly enhanced by the simultaneous treatment with other antitumor agents. This suggests that the combination of codeinone and other antitumor agents may produce only limited antitumor action, although the possibility still remains that other anticancer agents may cause more favorable effects with codeinone.

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