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Cell Death-inducing Activity of Opiates in Human Oral Tumor Cell Lines

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Abstract. *In screening cytotoxic agents in morphine alkaloids [**TE1-10**], codeinone [**TE8**] was cytotoxic against two human oral tumor cells lines (HSC-2 and HSG). The cytotoxic activity of codeinone (CC_{50}=1.0-1.2 μg/mL) against HSC-2 or HSG cells was higher than that of doxorubicin (CC_{50}=1.9-2.0 μg/mL). Human oral gingival fibroblasts (HGF) were relatively resistant to codeinone, as judged by higher SI ratio (3.7) suggesting the tumor-selective cytotoxicity of codeinone. The cytotoxic activity of morphine (CC_{50}=221 μg/mL) against HSC-2 was slightly lower than that of codeine (CC_{50}=186 μg/mL), thebaine (CC_{50}=125 μg/mL), etorphine (CC_{50}=94 μg/mL) or dihydroetorphine (CC_{50}=60 μg/mL). A study of structurally-related compounds suggested that the α,β-unsaturated ketone group of codeinone was responsible for its antitumor cytotoxicity. The cytotoxic activity of codeinone was significantly reduced by N-acetylcysteine, but not affected by $FeCl_3$, $CuCl_2$, $CoCl_2$, sodium ascorbate or catalase. Neither codeinone nor morphine inhibited P-glycoprotein-mediated rhodamine-123 efflux in multidrug resistant mouse T lymphoma L5178 transfected with human MDR 1 gene. These data suggest that codeinone induces cytotoxicity in oral tumor cell lines, possibly by a Michael-like addition of a protein SH or of an amino group to the bouble bond of codeinone.*

The morphine alkaloids are an important group of pharmaceuticals which have powerful analgesic properties. Recently, it has been shown that some opiates, such as morphine (1-3), etorphine (4) and buprenorphine (5, 6) are able to induce apoptosis in several cell lines. It is interesting that morphine has anticancer activity *in vitro* (2, 7). We were prompted to evaluate a series of morphine alkaloids for their antitumor cytotoxicity and here report our findings. Furthermore, we studied the MDR modulating activity of these morphine alkaloids on mouse lymhoma cells transfected with *MDR 1* gene, because an interaction of morphine and P-glycoprotein (Pgp) has been previously reported (8).

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Key Words: Codeinone, morphine alkaloid, cytotoxic activity, oral tumor cells, DNA fragmentation, MDR reversal.

Materials and Methods

Chemicals. The following chemicals were obtained from each indicated company: (±)-verapamil (Aldrich, Milwarkee, Wisconsin, USA); RPMI 1640 medium (GIBCo, Grand Island, NY, USA); ascorbic acid (Tokyo Kasei Kogyo Co. Ltd., Japan); fetal bovine serum (FBS) (JRH Biosci., Lenexa, KS, USA). Morphine hydrochloride [**TE10**] was purchased from Takeda Pharmaceutical Industries Co., Ltd. (Osaka, Japan). Codeinone [**TE8**] was synthesized from codeine [**TE7**] according to the literature (9). The compounds [**TE1-6** and **9**] were prepared from codeine [**TE7**] using previously documented procedures (10, 11).

Cell culture. Human promyelocytic leukemia (HL-60) cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, U.S.A.) in a humidified 5% CO_2 atmosphere. Human squamous cell carcinoma (HSC-2) cells and human salivary gland tumor (HSG) cells were maintained as monolayer cultures at 37°C in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere, then subcultured by trypsinization. Human gingival fibroblasts (HGF) were isolated from healthy gingival biopsies of a 10-year-old female, as described previously (12). Cells between the fifth and seventh passages were used.

Cytotoxic activity. The cells were incubated for 24 hours with the indicated concentrations of test samples in culture medium and the viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (12).

Assay for DNA fragmentation. The 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 4 mg/mL RNase A for 60 minutes at 50°C and 4 mg/mL proteinase K for 60 minutes at 50°C. DNA was extracted and precipitated by 70% ethanol, then dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0). DNA, equivalent to 5 x 10^6 cells, was applied to 1.8% agarose gel electrophoresis. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Cell and fluorescence uptake. MDR1/A expressing cell lines were selected

Table I. *Cytotoxic activity of morphine alkaloids against tumor and normal cells.*

Compound	Cytotoxic activity (CC_{50}, µg/mL)			
	HSC-2	HSG	HGF	SI (HGF/HSC-2)
TE1	125	93	181	1.5
TE2	125	156	178	1.4
TE3	140	170	>250	>1.8
TE4	120	115	>250	>2.1
TE5	60	98	188	3.1
TE6	94	63	149	1.6
TE7	186	68	218	1.2
TE8	1.0	1.2	3.7	3.7
TE9	213	98	>250	>1.2
TE10	221	170	>250	>1.1
(±)-Verapamil	51	28	43	0.8
Doxorubicin•HCl	2.0	1.9	>10	>5.0

HSC-2: Human oral squamous cell carcinoma cell line.
HSG: Human oral salivary gland tumor cell line.
HGF: Human oral gingival fibroblasts (5-7 population doubling levels).

by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype (13). The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a density of 2 x 10^6/mL and resuspended in serum-free McCoy's 5A medium, then a 0.5 mL aliquot of the cell suspension were distributed into each Eppendorf centrifuge tube. Next, 2 µL of 2 mg/mL test compounds were added and incubated for 10 minutes at room temperature. Subsequently, 50 µL rhodamine 123 (R123) as an indicator was added to the samples (5.2 µM final concentration) and the cells were incubated for a further 20 minutes at 37°C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) (pH 7.4) for analysis. The fluorescence of the cell population was measured by flow cytometry using the Beckton Dickinson FACScan instrument (cell sorter). (±)-Verapamil (**VP**) was used as the positive control in R123 accumulation experiments (14). The R123 accumulation was calculated from the fluorescence of one height value using the equation $y=10^{X/256}$. In the case of logarithmic transformation, the 1024 digital channels were switched to one decade at each 256 ($=2^8$) channels. Then, the percentage of mean fluorescence intensity was calculated in the parental and MDR cell lines, compared to the untreated cells. The fluorescence activity ratio was calculated by the following equation (14, 15):

MDR revarsal activity = (MDR treated/MDR control)/(parental treated/ parental control)

Results and Discussion

Cytotoxic activity. This study was designed to compare the cytotoxicity of several structurally-related opiate alkaloids

Table II. *Effects of additives on the cytotoxicity of codeinone (**TE8**) against HSC-2 cells.*

Additive	Cytotoxic activity (CC_{50}, µg/mL)
none	1.5
+ *N*-acetyl-L-cysteine (4 mM)	49.46
+ L-cysteine (4 mM)	5.40
+ sodium ascorbate (0.25 mM)	1.29
+ catalase (3000 U/mL)	1.02
+ $FeCl_3$ (0.2 mM)	1.72
+ $CoCl_2$ (0.2 mM)	1.55
+ $CuCl_2$ (0.2 mM)	1.13

HSC-2: Human oral squamous cell carcinoma cell line.

Table III. *Effects of morphine alkaloids on the multidrug resistance of L-5178 cells.*

Compounds	Concentration (µg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
Par (control)[b]	-	484	260.5	6068	43.3
MDR + R123 (mean)[c]	-	582.5	193.5	140.5	-
(±)-Verapamil	4	524	245	654	4.7
TE1	4	530	287	229	1.6
TE2	4	509	266	124	0.9
TE3	4	472	235	132	0.9
TE4	4	441	227	124	0.9
TE5	4	490	225	187	1.3
TE6	4	467	235	180	1.3
TE7	4	519	220	128	0.9
TE8	4	493	240	187	1.3
TE9	4	539	225	136	1.0
TE10	4	531	230	141	1.0

[a]FSC: Forward scatter count; SSC: Side scatter count; FL-1: Fluorescence.
[b]Par: a perental cell without MDR gene.
[c]MDR: a parental cell transfected with MDR gene.

(Figure 1) in human oral squamous cell carcinoma cell (HSC-2) line, human oral salivary gland tumor cell (HSG) line and human oral gingival fibroblasts (HGF). The opiates showed significantly varied cytotoxicity, depending on the structural features. As shown in Table I, the cytotoxic activity of these opiates against HSC-2 cells was: codeinone [**TE8**] (CC_{50}=1.0 µg/mL) > dihydroetorphine [**TE5**] (CC_{50}=60 µg/mL) > etorphine [**TE6**] (CC_{50}=94 µg/mL) > thebaine [**TE1**] (CC_{50}=125 µg/mL) > codeine [**TE7**] (CC_{50}=186 µg/mL) > morphine [**TE10**] (CC_{50}=221 µg/mL). The cytotoxic activity

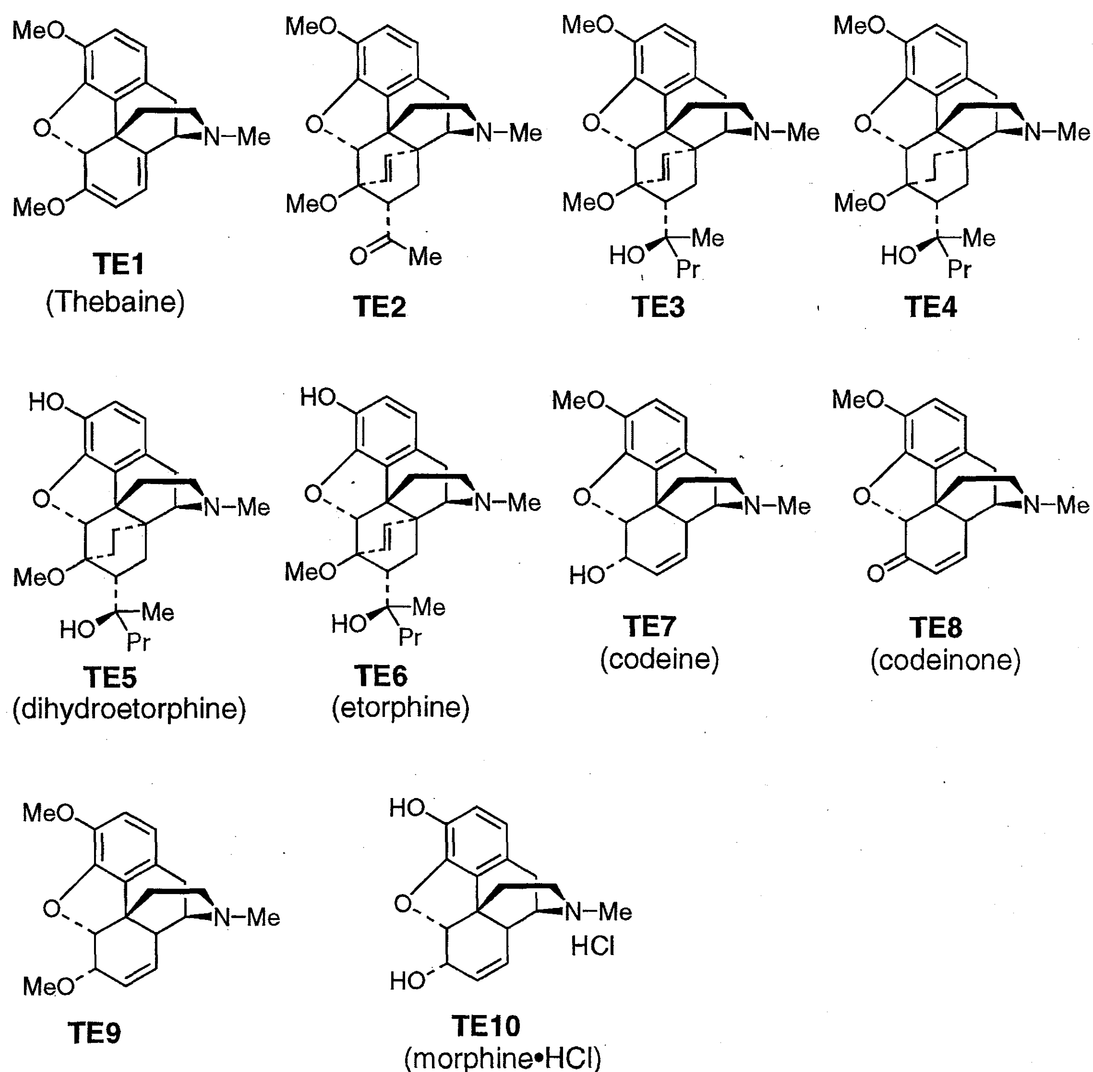

Figure 1. *Chemical structure of morphine alkaloids.*

of codeinone [**TE8**] (CC_{50}=1.0-1.2 μg/mL) against HSC-2 or HSG cells was higher than that of doxorubicine (CC_{50}=1.9-2.0 μg/mL). Normal fibroblasts (HGF) were relatively resistant to codeinone [**TE8**], as judged by higher SI ratio (3.7), suggesting the tumor-selective cytotoxicity of codeinone [**TE8**]. These selective actions add to their antitumor potential.

Codeinone is formed by the metabolism of codeine and is about thirty times more toxic than codeine (16). Glutathione (GSH) acts as a scavenger of codeinone (17). This is due to the fact that codeinone rapidly reacted *in vitro* with GSH, probably at the C-8 position by Michael addition, whereas codeine does not react with GSH (18). To clarify the mode of cell death induction by codeinone, the effect of codeinone on the internucleosomal DNA fragmentation characteristic of apoptosis was studied. Codeinone (4-8 μg/mL) elicited internucleosomal DNA cleavage, as shown by the agarose gel electrophoresis of DNA extracted from the treated HL-60 cells (Figure 2). A higher dose failed to induce DNA fragmentation, suggesting the presence of an optimal concentration for the induction of DNA fragmentation (Figure 2).

The effects of inhibitors on codeinone-induced cell death were also studied. The cytotoxic activity of codeinone was significantly reduced by *N*-acetylcysteine, but not affected by $FeCl_3$, $CuCl_2$, $CoCl_2$, sodium ascorbate or catalase (Table II).

Reversal of multidrug resistance (MDR) on tumor cells. MDR cells express the membrane glycoprotein (170 kDa) known as Pgp, which is an ATP-dependent active efflux pump. It has been demonstrated that the transport of morphine across the blood-brain barrier (BBB) is affected by Pgp both *in vitro* and *in vivo* (8). We measured the ability of opiates to inhibit Pgp-mediated R123 efflux in the MDR mouse T cell lymphoma

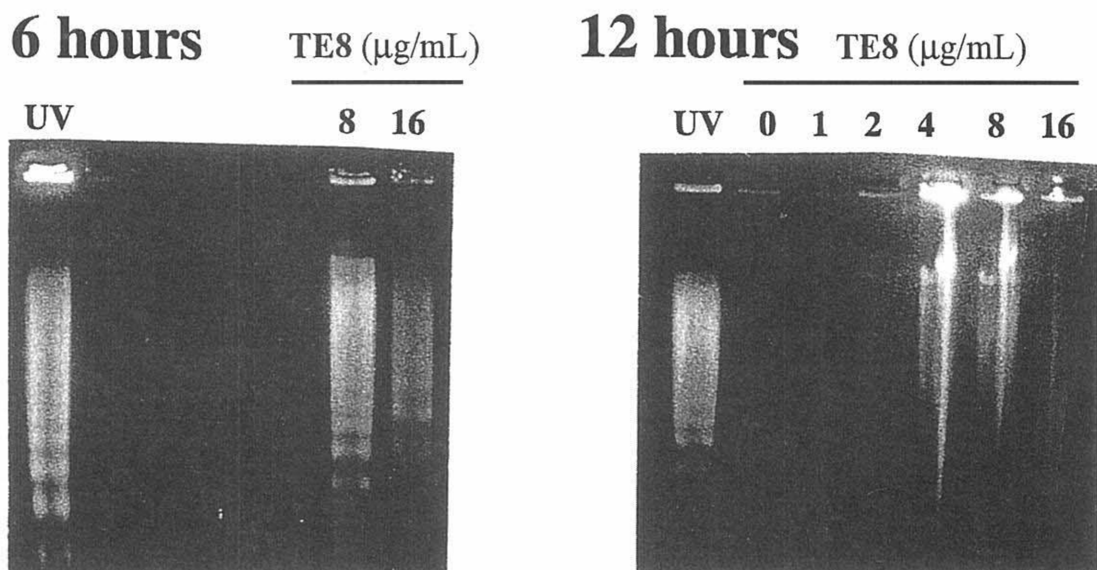

Figure 2. *Agarose gel analysis of DNA fragmentation of HL60 cells grown in the presence of codeinone (TE8).*

L5178 transfected with human *MDR 1* gene which was cultured in colchicine-containing medium (Table III). Addition of (±)-verapamil reversed the MDR, as reflected by the increase in R123 accumulation (fluorescence activity ratio (FR)=4.7) (Table III). Table III shows that most of the morphine alkaloids **[TE1-10]** failed to reverse MDR (FR=0.9-1.7), compared to higher MDR reversing activity of (±)-verapamil.

The present study demonstrated that the cytotoxic activity of the opiates was more pronounced tumor cell lines (HSC-2, HSG), as compared to against normal fibroblasts (HGF). The most potent codeinone had an α,β-unsaturated carbonyl group, which might contribute as a Michael acceptor in the structure. Thus, codeinone could offer an interesting model for the design of new compounds endowed with antitumor cytotoxicity.

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Received July 3, 2001
Accepted October 29, 2001