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# The Anticancer Mechanisms of Taxol- Diethylenetriamine pentaacetate Conjugate in HT29 Human Colorectal Cancer cells

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**Taxol, a natural product extracted from the *Taxus brevifolia,* is known to have significant anti-tumor activities against many common cancers, including ovarian and breast cancers. Despite the pronounced anti-tumor activity of this compound, its poor solubility in aqueous solutions hampers its clinical applications. We studied the anticancer mechanisms of the water-soluble taxol diethylenetriamine pentaacetate (DTPA) used for radiolabeling, and compared it to that of taxol. *In vitro* cytotoxicities of taxol and taxol-DTPA conjugate were tested in HT29 human colorectal cancer cells by the MTT method. As the result, the $IC_{50}$ value of the taxol-DTPA conjugate was about three fold higher than that of taxol. When analyzed by an agarose gel electrophoresis, the DNA ladders became evident after the incubation of cells with the taxol-DTPA conjugate for 24 h. We also found morphological changes of the cells undergoing apoptosis with electron microscopy. Next, we examined the signal pathway of taxol-DTPA conjugate-induced apoptosis in HT29 cells. The activation of extracellular signal-regulated protein kinase (ERK1/2) occurred at 10, 30, 60 and 120 min after 200 nM taxol-DTPA conjugate treatment. The pretreatment of the MEK inhibitor (PD98059) completely blocked the taxol-DTPA conjugate-induced ERK1/2 activation. The activated ERK1/2 translocated into the nucleus at the same time and phosphorylated its transcriptional factor, c-Jun. These results suggest that the taxol-DTPA conjugate has an apoptotic activity in HT29 cells, and that its proapoptic activity might be related with the signal transduction via ERK1/2 and c-Jun similar to that of taxol.**

**Keywords:** Taxol-DTPA, Taxol, Apoptosis, ERK1/2, c-Jun

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## Introduction

Paclitaxel (Taxol) is a potent antitumor drug that is remarkably effective against advanced ovarian and breast carcinoma. It also appears to be active against a variety of human tumor cell lines, including melanoma and colon adenocarcinoma (Riondel *et al.*, 1986). Taxol inhibits cell proliferation and induces cell death (Donaldson *et al.*, 1994; Milas *et al.*, 1995). It appears to act by slowing or blocking progression through mitosis (Long *et al.*, 1994; Liebmann *et al.*, 1994; Rieder *et al.*, 1994). Substoichiometric binding of taxol to reassembled microtubules potently stabilizes microtubule dynamics (Derry *et al.*, 1995). This binding acts as a radiosensitizer in the cells by inducing a sustained mitotic block at the metaphase/anaphase, which is sensitive in radiation (Hennequin *et al.*, 1996; Pulkkinen *et al.*, 1996).

Previous reviews (Wyllie *et al.*, 1980; Sen *et al.*, 1992; Fisher 1994) summarized the evidence that cancer chemotherapeutical agents, including taxol, induce apoptosis. Apoptosis is a regulated cell death process that is characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation (Kerr J.F.R. *et al.*, 1972). Apoptosis begins with the condensation of nuclear chromatin at the nuclear periphery, followed by blebbing of the nuclear and cytoplasmic membranes, and culminating in the fragmentation of the residual nuclear structure into discrete membrane-bounded apoptotic bodies (Allen 1987; Kerr *et al.*, 1987). The morphological alterations of apoptosis are accompanied by a variety of biochemical changes. Elevations in cytosolic free calcium (McConkey *et al.*, 1990), and the cytoplasmic hydrogen ion (Barry *et al.*, 1992), are followed by internucleosomal DNA degradation (Lockshin *et al.*, 1990; Arends *et al.*, 1991) and sharp decreases in cellular NAD levels (Berger *et al.*, 1987; Denisenko *et al.*, 1989). It was reported that the prolonged exposure of cells to low concentrations of taxol induced DNA fragmentation are characteristic of apoptosis, and ultimately lead to apoptosis (Liu *et al.*, 1994).

Recently, the mitogen-activated protein (MAP) kinase

cascade, which is well known for cell proliferation and the differentiation-inducing pathway, was reported to be associated with the apoptotic pathway (Kim *et al.*, 1996; Jeon *et al.*, 1998; Kim *et al.*, 1999). MAP kinase families include the extracellular regulated protein kinase (ERK 1/ERK 2), p38, and the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Molnar *et al.*, 1997; Raingeaud *et al.*, 1995). Well-studied members of the MAP kinase family are ERK1/2, which can be triggered by growth factors, and phorbol esters through the Ras-dependent activation of the Raf-MEK-ERK pathway (Davis *et al.*, 1993; Stoke *et al.*, 1994). The JNK cascade is operated by a parallel signaling module consisting of the MEKK-1/ SEK 1/ JNK cascade (Kyriakis *et al.*, 1994; Coso *et al.*, 1995). Unlike ERK, the JNK is strongly activated by environmental stresses, such as inflammatory cytokine (Martin *et al.*, 1997), ultraviolet C (Kyriakis *et al.*, 1994), gamma irradiation (Yu *et al.*, 1996), and a DNA-damaging drug (Jimenez *et al.*, 1997). In addition, the phosphorylated MAP kinase in cytosol is translocated into nucleus (Chen *et al.*, 1992). It is also involved in the regulation of transcription factors, including c-Jun, c-fos and c-Myc, which have been shown to influence apoptosis (van Dam *et al.*, 1995; Cavigelli *et al.*, 1995; Baek *et al.*, 1996). Recently, It was reported that taxol activated ERK1/2 and c-Jun in the cervical carcinoma cell line HeLa (Kim *et al.*, 1999), JNK, and p38 in the ovarian cancer cell line OVCA 420 (Lee *et al.*, 1998), as well as in the breast cancer cell MCF-7 (Shtil *et al.*, 1999).

Despite its anti-tumor activity, taxol's aqueous insolubility (<0.004 mg/ml) hampers its clinical applications. Protaxols, taxol-releasing compounds, and taxol derivatives were designed for their aqueous solubility (Nicolaou *et al.*, 1993). And the biodistribution and imaging properties of $^{111}$In-DTPA-taxol were investigated (Li *et al.*, 1997; Inoue *et al.*, 1999). Diethylenetriamine pentaacetic acid (DTPA) is often used as a chelating agent for radiolabeling.

We studied the anticancer mechanisms of the taxol-DTPA conjugate (Fig. 1), which could be used for radiolabeling as it has a high water solubility, compared to that of taxol. In this report, we examined the cytotoxicity and apoptotic effect of the taxol-DTPA conjugate in HT29 human colorectal cancer cells. In particular, the potent activation of ERK ½, and its nuclear translocation, were observed after the taxol-DTPA conjugate treatment. The significance of these observations has been discussed.

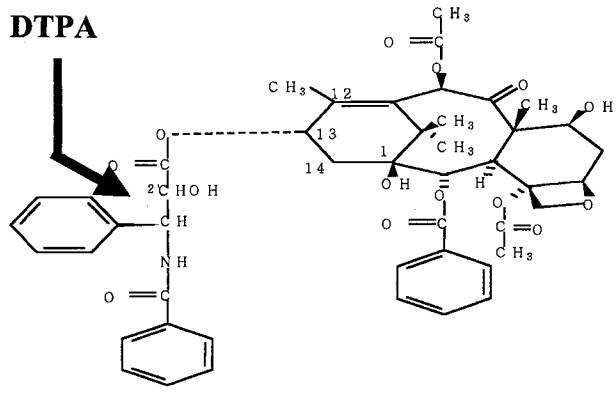



**Fig. 1.** Structure of the taxol-DTPA conjugate.

## Materials and Methods

**Materials** The paclitaxel (taxol) was purchased from the Sigma Chemical Co.(St. Louis, USA). The taxol-DTPA conjugate was provided by Dr. O.D.Awh (University of Yonsei, Wonju). The anti-phospho-ERK1/2, anti-phospho-c-Jun antibodies, and MEK 1 inhibitor (PD98059) were purchased from New England Biolabs (Beverly, MA). All other chemicals and reagents were of the highest grade commercially available.

**Cell culture and *in vitro* cytotoxicity assay** HT29 human colorectal cancer cells were maintained in DMEM that was supplemented with 10% fetal bovine serum without antibiotics, and an antifungal agent. All of the cells were grown at 37°C in a humidified atmosphere of 5% $CO_2$. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. Exponentially growing cells were inoculated to $5 \times 10^3$ cells/well using a 96 well microplate that was supplemented with 100 µl DMEM. For experiments that studied cells in the plateau phase of growth, the cells were permitted to grow for a minimum of 72 h before they were exposed to drugs. The cells were exposed to various concentrations of taxol, DTPA, and a taxol-DTPA conjugate. DMSO was used to dissolve taxol, DTPA, and the taxol-DTPA conjugate. The final concentrations of DMSO in the cell medium were less than 0.01%. This concentration of DMSO had no effect on the cell growth, as determined by the control experiments. After the treated cells were incubated for 48 h, 50 µl MTT (1 mg/ml, Sigma) was added, and the plates were incubated at 37°C for 4 h. To dissolve formazan, 150 µl DMSO was added, and the plates were measured at 540 nm by a spectrometer. The $IC_{50}$ values were determined by plotting of the drug concentration versus the survival ratio of the treated cells.

**DNA extraction and electrophoresis** Five million cells, which were treated with the taxol-DTPA conjugate and taxol for 24 h, were washed twice in a solution of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, and then they were lysed with a 500 µl lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA). Lysates were harvested by 1,000 × g for 10 min and the supernatants were incubated for 3 h at 37°C with 50 µg/ml RNase A, 120 µg/ml proteinase K. Then, phenol/chloroform/isoamylalcohol (25 : 24 : 1, Sigma) extracted DNA. After precipitation, the pellets were resuspended in a 30 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through a 1.8% agarose gel that contained ethidium bromide. The gel was then visualized by UV fluorescence.

**Electron microscopy** The cells, which were treated with 200 and 400 nM taxol-DTPA conjugate and 50 and 100 nM taxol for 24 h, were centrifuged at 400 × g, fixed with 2% glutaraldehyde in PBS for 24 h, washed in 0.1 M Caocodylate, pH 7.4, and fixed with

0.1% $OsO_4$ in 0.1 M Caocodylate for 1 h 30 min. After fixation, the cells were washed with in 0.1 M Caocodylate, pH 7.4, and then dehydrated in graded ethanol. Next, the cells were impregnated with propylene oxide and embedded in Polybed 812 (Polyscience, Inc., Warrington, PA). After a 60°C incubation, the cells were cut, and stained with uranyl acetate and lead citrate.

**Preparation of cytosolic and nuclear fractions** To examine the activation of ERK1/2, exponentially growing cells were starved for 24 h, then exposed to 200 nM taxol-DTPA conjugate and 50 nM taxol for 10, 30, 60 and 120 min. To analyze the effects of PD98059 on the taxol-DTPA conjugate and taxol-treated cells, the starved cells were exposed to 10 μM PD98059 for 1 h, then treated with 200 nM taxol-DTPA conjugate and 50 nM taxol for 10, 30, 60 and 120 min. The treated cells were washed twice and collected into 1 ml hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 40 μg of PMSF per ml and 10 μg of both pepstain and leupeptin per ml, pH 7.5). The cellular suspensions were homogenized and harvested by 600 × g for 5 min. To prepare the cytosolic fraction, the supernatant was obtained and centrifuged at 12,000 × g for 20 min. The pellets were suspended by 0.25 M sucrose in 1.5% citric acid and passed three times through a 26 gauge needle and loaded onto 1 ml of 0.88 M sucrose cushion in 1.5% citric acid. To get the nuclear fraction, following centrifugation at 900 × g for 10 min, the pellets were dissolved with a hypotonic lysis buffer containing 0.5% Igepal CA-630, 0.1% deoxycholate, 0.1% Brij-35, and then centrifuged at 10,000 g for 10 min. All of the fractional procedures were completed on ice.

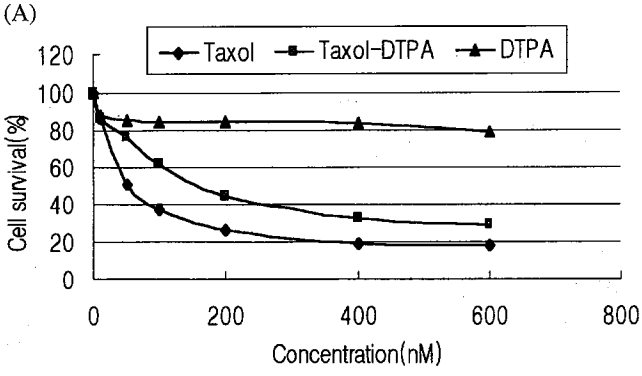
The purity of the nuclear fraction was determined by measuring the lactate dehydrogenase activity as the cytosol marker. Then, the nuclear fractions were confirmed by a Western blot analysis.

**Immunoblot analysis** For a ERK1/2 immunoblot analysis, we first examined the protein concentrations of the prepared cytosolic and nuclear extracts with a Bradford assay solution (100 mg/L Coomassie Brilliant Blue G-250, 50 ml/L 95% ethanol, 100 ml/L 85% phosphoric acid). The same concentrations of protein samples were electrophoresed on a 10% polyacrylamide gel in the presence of SDS. After electrophoresis, the proteins were transferred to nitrocellulose membranes that were blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) containing 10% skim milk. Then, the membranes were incubated with anti-phospho-ERK1/2, anti-phospho-c-Jun antibodies at adequate dilutions in TBS for 1 h at room temperature. After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse and anti-sheep IgG antibodies at a 1 : 1,000 dilution for 1 h at room temperature (washed 3 times in TBST). They were then detected with an enhanced chemiluminescence detection method by immersing the blots for 1 min in a 1 : 1 mixture of chemiluminescence reagents A and B (Amersham, UK), and exposing them then to Kodak film for a few minutes.

## Results and Discussions

***In vitro* cytotoxic effects** Exponentially growing cells were exposed to various concentrations of taxol, DTPA, and the taxol-DTPA conjugate. After 48 h of exposure to taxol, low concentrations (10 nM to 100 nM) of the drug resulted in a steep decline in cell survival, but is, on the other hand, flat over a wide range of taxol concentrations. Increasing the DTPA concentrations, however, resulted in no additional cytotoxicity. Increasing the taxol-DTPA conjugate concentrations gradually decreased cell survival (Fig. 2A). The minimal concentrations of the taxol-DTPA conjugate, and taxol to inhibit the growth of HT29 cells by 50% ($IC_{50}$), were determined. The $IC_{50}$ value of the taxol-DTPA conjugate had 168.2 nM, which is about three fold higher than that of taxol (Fig. 2B). Taxol has two hydroxyl groups that can be most conveniently functionalized: C-2' and the C-7-hydroxyl group (Nicolaou *et al.*, 1993: Li *et al.*, 1997). Previous studies have shown that the side chain of C-13 has a cytotoxicity (Li *et al.*, 1997: Mathew *et al.*, 1992). This data resulted from our use of taxol conjugate, in which the C-2' hydroxyl group in the side chain of C-13 was substituted with DTPA.

(A)




(B)

| Drugs | Taxol-DTPA | Taxol |
|---|---|---|
| $IC_{50}$ value (nM)* | 168.2 | 52.3 |

*$IC_{50}$ value is the drug concentration when cell growth inhibition is 50%.

**Fig. 2.** Cytotoxic effects of the taxol-DTPA conjugate, DTPA and taxol in HT29 cells.

**DNA fragmentation** In order to determine the apoptotic effect of the taxol-DTPA conjugate, we examined the apoptotic response, as judged by the appearance of a DNA ladder by a 1.8% gel electrophoresis at various concentrations of the taxol-DTPA conjugate and taxol. The DNA ladder patterns with taxol-DTPA conjugate began to appear at 1nM concentrations, similar to taxol (Fig. 3). The ladders were also observed at 200 and 400 nM taxol-DTPA conjugate (data not shown). Thus, while the taxol-DTPA conjugate and taxol exhibited DNA fragmentations in a broader range of concentrations in the HT29 cells, leukemia cells produced the classical internucleosomal DNA fragmentation of apoptosis when exposed to 100 nM taxol for 24 h (Tang *et al.*, 1994).

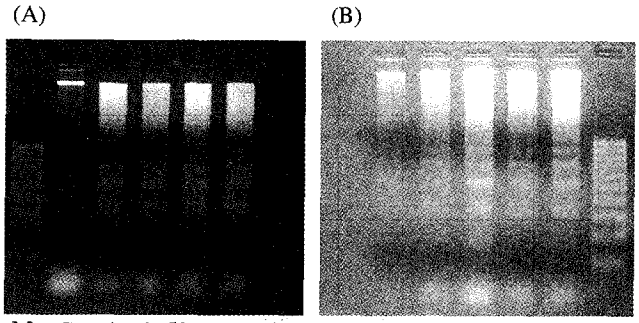



**Fig. 3.** DNA fragmentations extracted from HT29 cells treated with the taxol-DTPA conjugate (A) and taxol (B) for 24 h.

**Electron Microphotography** When the condensed nuclei were fixed with glutaraldehyde, and examined by a thin section EM, the treatment of the HT29 cells with the taxol-DTPA conjugate and taxol, resulted in morphological changes that are consistent with the process of apoptosis. Initially in the nucleus, a rim of heterochromatin appeared at the nuclear periphery, and the nucleolus simultaneously disappeared (Fig. 4B,C,D). In this case, mitochondria and the plasma membrane remained intact throughout the course of these morphological changes. Consequently, ~50% of the cells continued to exclude trypan blue. Then, the nuclei subsequently fragmented (Fig. 4C, E). Finally, few were found after packaging of the nuclear fragments into multiple membrane-enclosed apoptotic bodies. It was reported that HeLa cells showed apoptotic bodies when the cells were treated with 50 nM taxol for 24 h (Kim *et al.*, 1999).

**Activation and translocation into nucleus of ERK1/2** It is known that the MAP kinase pathway is involved in the apoptotic signal transduction (Wang *et al.*, 1998). To determine if ERK1/2, one member of MAP kinase family, were activated by the taxol-DTPA conjugate stimulation of inducing an apoptotic response, we examined the phosphorylation of ERK1/2 by an immunoblot analysis. HT29 cells were exposed with 200 nM taxol-DTPA conjugate

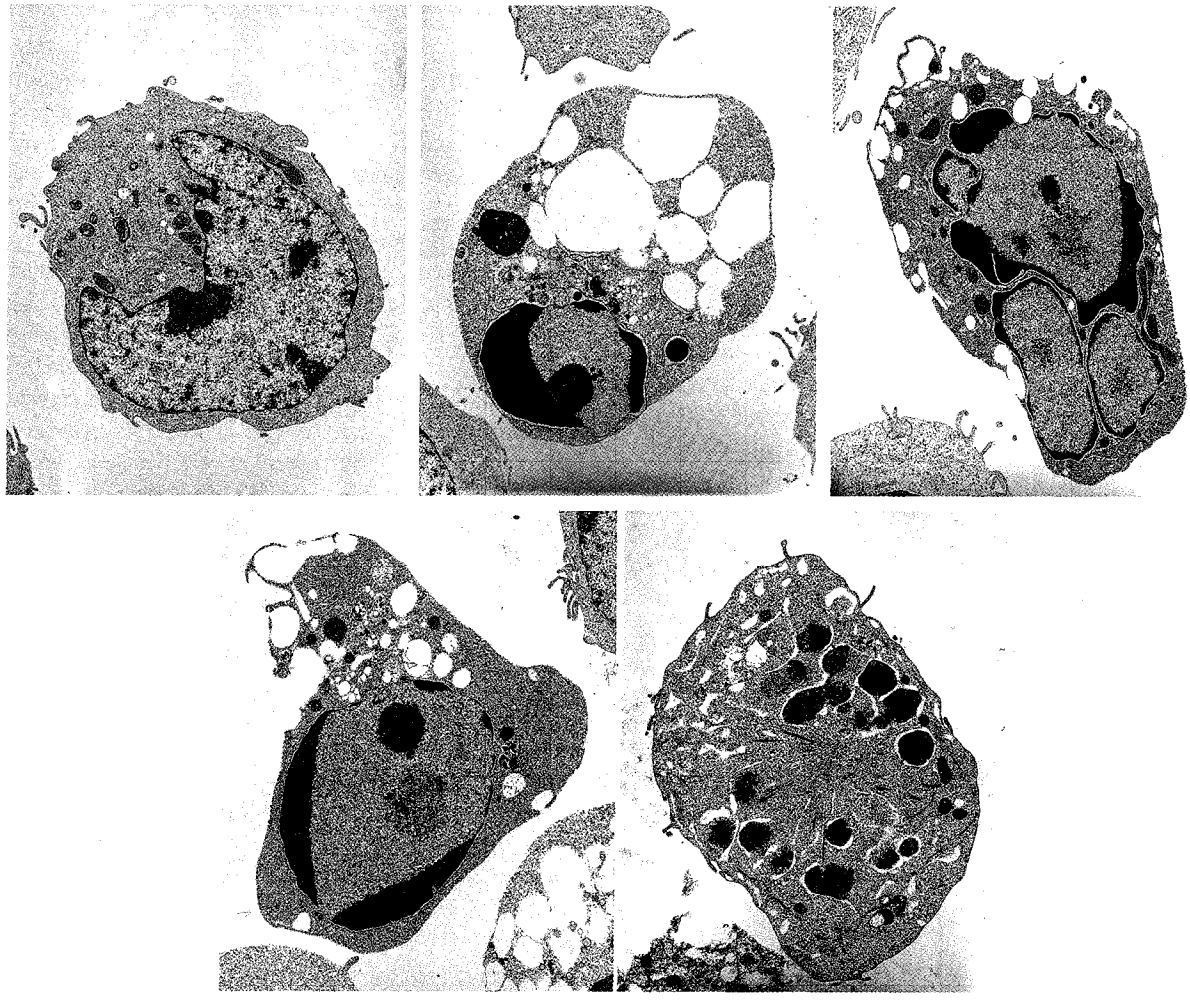


**Fig. 4.** Electron micrographs in HT29 cells treated with the taxol-DTPA conjugate (B, 200 nM; C, 400 nM) and taxol (D, 50 nM; E, 100 nM) for 24 h. Control (A).

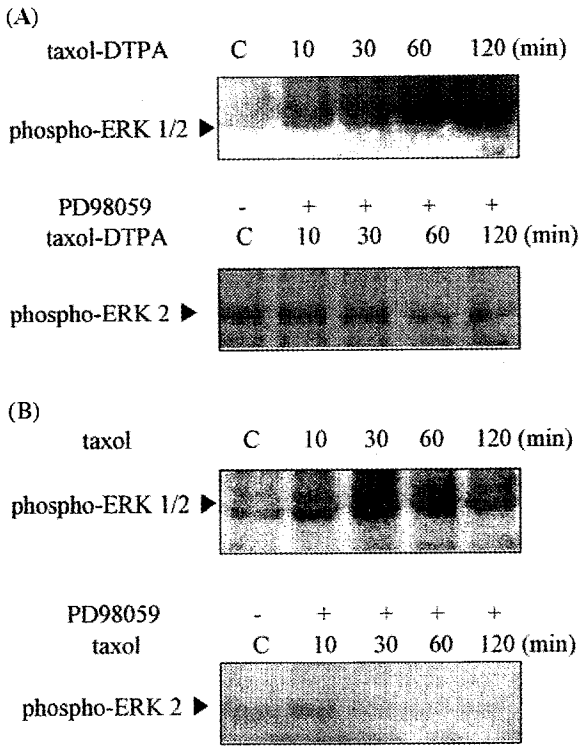



**Fig. 5.** Immunoblotting analysis of ERK1/2 activation by the taxol-DTPA conjugate and taxol, and the inhibition of phospho-ERK1/2 by PD98059. (A) HT29 cells were treated with 200 nM taxol-DTPA conjugate (upper), and HT29 cells were treated with 10 μM PD98059 for 1 h and then treated with 200 nM taxol-DTPA conjugate (lower) for 10, 30, 60 and 120 min (lane 2-5), serum free control (lane 1). (B) HT29 cells were treated with 50 nM taxol (upper) and HT29 cells were treated with 10 μM PD98059 for 1 h, and then treated with 50 nM taxol (lower) for 10, 30, 60 and 120 min (lane 2-5), serum free control (lane 1).

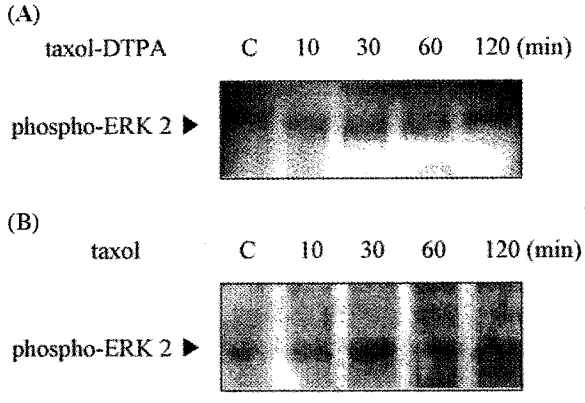



**Fig. 6.** Immunoblotting analysis of phospho-ERK1/2 translocation from cytosol to nucleus. HT29 cells were treated with 200 nM taxol-DTPA (A) and 50 nM taxol (B) for 10, 30, 60 and 120 min (lane 2-5), serum free control (lane 1).

for 10, 30, 60 and 120 min. The result was that the taxol-DTPA conjugate activated ERK1/2 at 10, 30, 60 and 120 min after treatment. When the cells were exposed to 200 nM, the

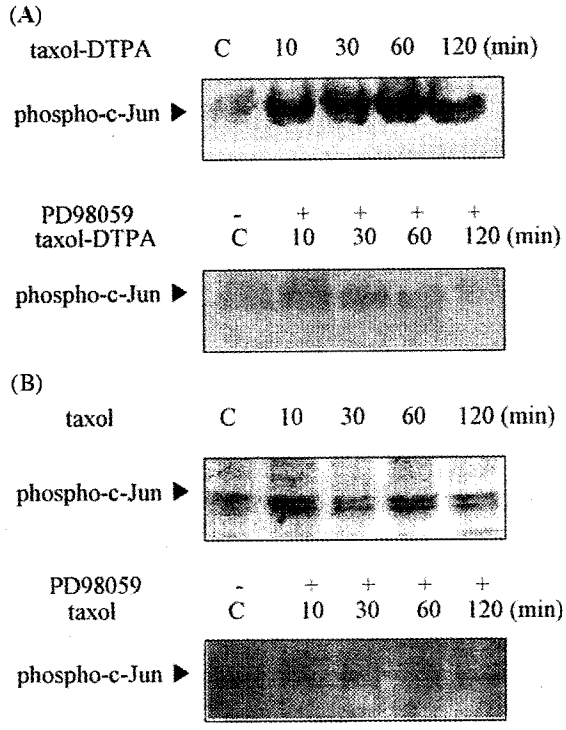



**Fig. 7.** Immunoblotting analysis of phospho-c-Jun by the taxol-DTPA conjugate, and taxol and the inhibition of phospho-c-Jun by PD98059. (A) HT29 cells were treated with 200 nM taxol-DTPA conjugate (upper), and HT29 cells were treated with 10 M PD98059 for 1 h, then treated with 200 nM taxol-DTPA conjugate (lower) for 10, 30, 60 and 120 min (lane2-5), serum free control (lane 1). (B) HT29 cells were treated with 50 nM taxol (upper), and HT29 cells were treated with 10 μM PD98059 for 1 h, then treated with 50 nM taxol (lower) for 10, 30, 60 and 120 min (lane2-5), serum free control (lane 1).

taxol-DTPA conjugate in the presence of 10 μM PD98059, a specific inhibitor of the MAP kinase kinase (MEK), the activation of ERK1/2 did not occur (Fig. 5). It has been proposed that the prolonged activation of the MAP kinase is accompanied by the translocation of the enzyme to the nucleus (Chen *et al.*, 1992) with subsequent alterations in the gene expression (Marshall *et al.*, 1995). To confirm the nuclear translocation of ERK1/2, we prepared the separation of nuclei from the cells exposed with 200 nM taxol-DTPA conjugate for 10, 30, 60 and 120 min and examined it with an immunoblot analysis. As shown in Fig. 6, ERK1/2 (44/42kD) was phosphorylated and translocated into nucleus by the taxol-DTPA conjugate at the same condition of inducing apoptosis. Accordingly, ERK1/2 may be involved in the signal transduction of the taxol-DTPA conjugate-induced apoptosis.

**c-Jun phosphorylation** To study the possible regulation of the nuclear target by the taxol-DTPA conjugate, the nuclear fractions from 200 nM taxol-DTPA conjugate-treated HT29 cells were examined for the activity of transcriptional factor, c-Jun, by immunoblot with phospho-c-Jun Ab. The result was

that the taxol-DTPA conjugate activated c-Jun comparatively at 10, 30, 60 and 120 min after treatment. It was reported that 50 nM taxol activated c-Jun at 10 and 20 min in HeLa cells (Kim *et al.*, 1999). When the cells were exposed to 200 nM taxol-DTPA conjugate in the presence of 10 μM PD98059, the activation of c-Jun does not occur (Fig.7). Therefore, this results suggests that c-Jun is a downstream effector of ERK1/2 activation that is responsive to treatment with the taxol-DTPA conjugate.

In summary, the taxol-DTPA conjugate has an antitumor activity against HT29 human colorectal cancer cells and induces the apoptotic response. Also, our results imply that the activation and translocation into the nucleus of ERK1/2 may be related to the apoptotic signal transduction of the taxol-DTPA conjugate. It also suggests that c-Jun has a downstream effector that is responsive to treatment with the taxol-DTPA conjugate, and its anticancer mechanism might be similar to that of taxol.

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