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# Apoptosis Inducing Effects of 6-Methoxydihydrosanguinarine in HT29 Colon Carcinoma Cells

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6-Methoxydihydrosanguinarine (6ME), a benzophenanthridine alkaloid derived from the methanol extracts of *Hylomecon hylomeconoides*, showed a dose-dependent effect at 1-10 μM on causing apoptotic cell death in HT29 colon carcinoma cells ($IC_{50}$ = 5.0 ± 0.2 μM). Treatment of HT-29 cells with 6ME resulted in the formation of internucleosomal DNA fragmentation. Treatment of the cells with 6ME caused activation of caspase-3, -8 and 9 protease and subsequent proteolytic cleavage of poly(ADP-ribose)polymerase. 6ME increased the expression of p53 and Bax and decreased the expression of Bid. These results indicate that p53 and proapoptotic Bcl-2 family proteins might participate in the antiproliferative activity of 6ME in HT29 cells.

**Key words:** 6-Methoxydihydrosanguinarine, *Hylomecon hylomeconoides*, Apoptosis, HT29 cells

## INTRODUCTION

Apoptosis is a genetically programmed form of cell death, which involves the activation of an endogenous suicide program. Apoptosis is vital for normal physiological processes that are also involved in a number of pathological conditions such as autoimmune diseases, cancer, neurodegenerative disease, and AIDS (Devitt *et al.*, 1999). Improvements in surgical and adjuvant therapies and in dietary and screening programs have facilitated an overall decline in the mortality of colon cancer. Nevertheless, colorectal cancer accounts for 11% of all cancers in the United States, which is not much different in our case. In the year 2001, the incidence rate of colon cancer is 10.5% and the death rate is 7.7% (Greenlee *et al.*, 2001). In recent years, several important advances have been made in understanding the biology and genetics of colorectal cancer, and new evidence has linked the use of chemopreventive agents with the decreased incidence of colorectal cancer. Among them, the cyclooxygenase inhibitors have been attracted considerable attention for the prevention of the type of cancer. Several randomized intervention trials of a variety of cyclooxygenase inhibitors in patients with familial adenomatous polyposis have shown that these medications are effective in not only preventing incident adenomas, but in contributing to the regression of existing lesion (Giardiello *et al.*, 1993; Steinbach *et al.*, 2000).

6-Methoxydihydrosanguinarine (6ME) is an isoquinoline alkaloid derived from the methanol extracts of *Hylomecon hylomeconoides*. 6ME was found to possess anti platelet aggregation activity (Chen *et al.*, 2001). It has also been reported to have antiproliferative effects on K549 human lung cancer cells, PC3 human prostate cancer cells, MCF-7 human breast cancer cells and A562 human leukemia cells (Cho, 2001).

In the present study, we examined the effects of 6ME on the inhibition of proliferation and the induction of apoptosis in human colon carcinoma HT29 cells. We found that 6ME suppresses the proliferation and induces apoptosis in HT29 cells.

## MATERIALS AND METHODS

### Materials and cell culture

6-Methoxydihydrosanguinarine (C43 or 6ME) and dihydrosanguinarine (C44) are derived from the methanol extracts of *Hylomecon hylomeconoides* and lycorine (C41) and crinamine (C42) are from *Crinum asiticum var*

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*japonicum*, all of which have isoquinoline alkaloid in their structure (Fig. 1). The human colon carcinoma cell line, HT-29 was maintained in the logarithmic phase of growth in DMEM medium (GIBCO BRL, Grand Island, NY) supplemented with heat inactivated 10% fetal bovine serum (GIBCO BRL) at 37°C in a 5% $CO_2$-95% air humidified incubator. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was obtained from Sigma Chemical Co. All other chemicals used were of the highest pure grade available.

### Cell viability assay

At the end of 0, 3, 6, 12 h incubation period MTT assay was performed to determine the viability of the cells (Monks *et al.*, 1991).

### DNA fragmentation analysis

The cells grown at a density of $2\times10^6$ cells/mL in 100 mm culture dishes were treated with 6-ME as described in the figure legends. Genomic DNA was prepared with Wizard Genomic DNA Purification Kit (Promega, Madison, WI). DNA was precipitated with isopropanol, separated in 1.5% agarose gel and visualized by UV illumination after ethidium bromide staining.

### Measurement of caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC; caspase-3 substrate), *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC; caspase-8 substrate) and *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (Ac-LEHD-AMC; caspase-9 substrate). Each substrate was added to the cell lysates in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, pH 7.4) and incubated for 3 h at 37°C. The cleavage of the peptide substrate was monitored at excitation wavelength at 380 nm and emission at 460 nm. Results are normalized to protein content and expressed as the percent change of the activity compared to the untreated control.

### Western blot analysis

Cells were washed with PBS and lysed (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 50 mM β-glycerophosphate, 20 mM NaF, 1 mM $Na_3VO_4$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Cell lysates were centrifuged and the protein content was determined. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (12-15%), transferred to nitrocellulose membrane and immunoblotted with antibodies as indicated. Detection was performed using enhanced chemiluminescence Western Blotting Detection Reagents (Amersham). Monoclonal human anti-poly(ADP-ribose) polymerase (PARP) (1:1,000 dilution), polyclonal human anti-caspase-3 (1:1,000), polyclonal human anti-caspase-9 (1:1,000), and polyclonal human anti-p53 antibody were purchased from Cell Signaling Tech. (Beverly, MA). Monoclonal human anti-Bcl-2 (1:250), anti-Bid (1:250) and anti-Bax (1:250) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

## RESULTS

### Effects of 6ME on cell proliferation

Inhibition of cell proliferation was tested by MTT assay following treatment of HT29 cells with the test compounds (Fig. 1). The number of viable cells was determined at each time point indicated in Fig. 2. C43 (6ME) was the most potent inhibitor of HT29 cell proliferation. 6ME decreased cell proliferation significantly in dose- and time-dependent manner. The concentration required for 50% inhibition of the growth ($IC_{50}$) was 5.0 ± 0.2 μM in HT29 cells at 6 h (Fig. 2B).

### Induction of apoptosis by 6ME

To clarify the mode of cell death caused by 6ME, we examined the effects on the internucleosomal DNA fragmentation, which is a characteristic feature of apoptosis. As shown by the agarose gel electrophoresis, increased DNA fragmentation was apparent in HT29 cells after treatment with 5 μM 6ME for 6 and 12 h (Fig. 3A).

### Caspase activity and PARP cleavage

Caspase-3 activation is a late signal that accomplishes apoptosis in mammalian cells. To determine whether caspase activation is involved in the 6ME-induced cell

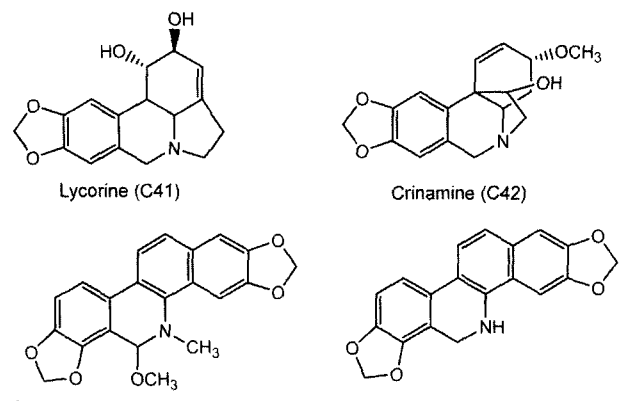


**Fig. 1.** Structure of lycorine (C41) and crinamine (C42) derived from the methanol extract of *Crinum asiticum var japonicum*, and 6-methoxydihydrosanguinarine (C43 or 6ME) and dihydrosanguinarine (C44) from *Hylomecon hylomeconoides*.

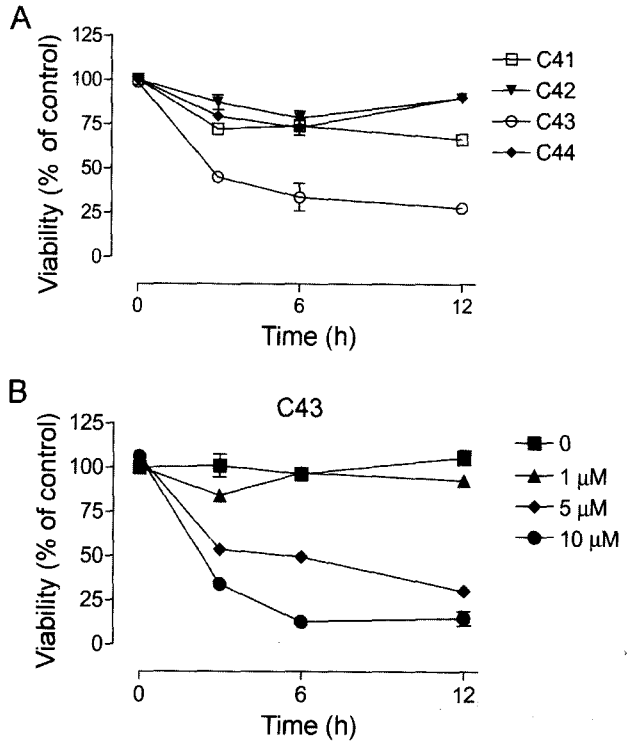


**Fig. 2.** Time and dose-dependent inhibition of HT29 cell growth. Cells were incubated with (A) 5 μM of C41-C44 or (B) with 0 10 μM C43 (or 6ME) for the indicated time and cell viability was determined by MTT assay. Data are mean±S.D. of three independent experiments and represented as % changes compared to the vehicle treated control at time 0.

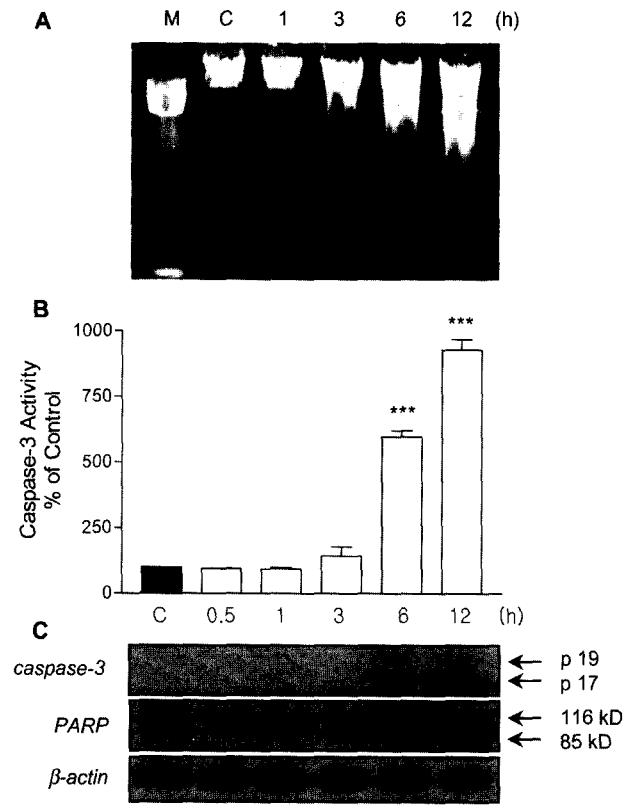


**Fig. 3.** 6ME induces apoptosis in HT29 cells, which involves the activation of caspase-3 and subsequent cleavage of PARP. (A) DNA fragmentation after exposure of HT29 cells to 6ME (5 μM). Genomic DNA was prepared and separated in 1.5% agarose gel and visualized by UV illumination after ethidium bromide staining. (B) Activation of caspase-3 by 6ME (5 μM). Extracts of control cells and cells treated with 6ME were analyzed for caspase activity as a function of concentration. Ac-DEVD-AMC was used as the substrates for caspase-3, and the cleavage of each peptide was monitored at excitation wavelength at 380 nm and emission at 460 nm. Each data point represents the mean±S.D. from three independent experiments (***$P<0.001$). (C) Representative Western blots showing the cleavage of caspase-3 and PARP after treatment with 6ME (5 μM). Cells were treated with 6ME, and total cellular protein was separated on a 12% SDSpolyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-caspase-3 and anti-PARP antibody.

death, HT29 cells were exposed to 6ME and assayed for caspase-3 activity. Treatment of the cells with 6ME activated caspase-3 in a time dependent manner (Fig. 3B and C). Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Treatment of HT29 cells with 5 μM of 6ME caused a time-dependent proteolytic cleavage of PARP (Fig. 3C). Caspase-8 and 9 activities were also increased in a time-dependent manner (Fig. 4).

### Apoptosis-associated proteins

Death-promoting members of the Bcl-2 family, such as Bax and Bid, play key roles in the chemical-induced apoptosis. Bid and Bax in the cytosol receive death signals from upstream events and induce the release of cytochrome c, thereby activating the mitochondrial apoptotic pathway. Fig. 4A shows details of the levels of Bid, Bax, and p53 in whole-cell lysates of HT29 cells treated with 6ME. The level of Bax was increased in a time-dependent manner, and that of full length Bid was decreased, indicating the possibility of the release of truncated Bid. P53, a transcription factor of Bax was also up-regulated in 6ME-treated cells (Fig. 5).

## DISCUSSION

The study presented here demonstrates that 6-methoxydihydrosanguinarine, an isoquinoline type alkaloid isolated from the methanol extracts of *Hylomecon hylomeconoides*, inhibits proliferation and induces apoptosis in human colon carcinoma HT29 cells. Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated, or activated in response to specific stimuli or various forms of cell injury. In cancer biology, it is now evident that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self-destruction. Therefore, it would be advantageous in

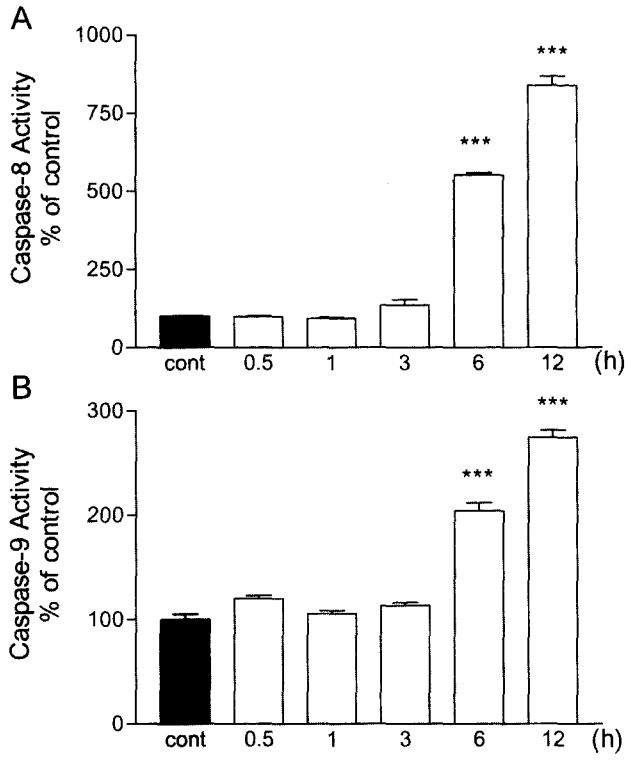


**Fig. 4.** Activation of caspase-8 and 9 were monitored using Ac-IETD-AMC and Ac-LEHD-AMC, respectively. Each data point represents the mean ± S. D. from three independent experiments (*** $P < 0.001$).

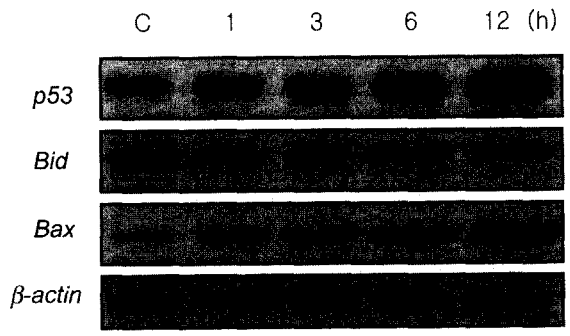


**Fig. 5.** 6ME (5 μM) induces expression of p53 and Bax. Representative Western blots showing the time-dependent up-regulation of p53 and Bax. HT29 cells were treated with 6ME and the protein from the whole-cell lysate was separated on a 12% SDSpolyacrylamide gel.

cancer chemotherapy and prevention to tip the balance in favor of apoptosis over mitosis.

6ME was found to possess anti platelet aggregation activity (Chen *et al.*, 2001). It has also been reported to have antiproliferative effects on K549 human lung cancer cells, PC3 human prostate cancer cells, MCF-7 human breast cancer cells and A562 human leukemia cells (Cho, 2001). Based on these reports, we hypothesized that 6ME may have an impact on cancer cells in terms of proliferation and apoptosis. In the present study, we show that 6ME inhibits the growth of human colon carcinoma cells and induces apoptosis, which is associated with the proteolytic degradation of PARP via activation of caspase-3. Although the molecular mechanism underlying the action of 6ME in tumor cells is not fully understood, these data may have a direct or indirect bearing on the tumor cell growth-inhibiting activity of this compound.

A central component of the apoptotic process is a proteolytic system involving the caspases, a highly conserved family of cysteine proteases with specific substrates. Caspase 8 represents the apical caspase in the death receptor (extrinsic) pathway and caspase 9 serves as the apical caspase of the mitochondrial (intrinsic) pathway (Korsmeyer *et al.*, 2000; Juo *et al.*, 1998). Caspase 3 has been shown to play an important role in apoptosis induced by several conditions, and to be necessary in determining the nuclear alteration of apoptosis (Janicke *et al.*, 1998). It is commonly believed that caspases with long prodomains are upstream or initiating caspases whereas those with short prodomains are effectors or executioner caspases. For example, caspase 8 is the most proximal caspase to become activated upon ligation of the Fas molecule since this caspase is directly recruited into the Fas signaling complex upon receptor aggregation (Kischkel *et al.*, 1995; Muzio *et al.*, 1996). In the present study, we demonstrated that a series of caspases are activated by treatment with 6ME, which indicates that 6ME-induced cell death is under control of cellular death machinery. 6ME activates various signaling molecules and apoptosis-related genes. P53 protein is a critical mediator of cellular responses to DNA damage in mammalian cells. It exerts its function by arresting the G1 phase of the cell cycle and mediates apoptosis in cells exposed to anti-cancer drugs (Lowe *et al.*, 1993). P53 exerts its effects on cells as a transcription factor for Bax, PIG genes, CD95, and DR5 (Herr and Debatin, 2001). In this case, an increase in p53 leads to the expression of pro-apoptotic proteins, which prompt cells to undergo apoptosis.

In summary, in the present study we propose a growth-inhibitory effect for 6ME derived from *H. hylomeconoides* against HT29 cells that acts at least partially through the induction of apoptosis. These results suggest that the compound may have a potential use as a chemopreventive and/or chemotherapeutic agent.

## ACKNOWLEDGEMENT

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