

Apoptotic activity of a new jasmonate analogue is associated with its induction of DNA damage

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Abstract. The current study was undertaken to investigate the effects of methyl 5-chloro-4,5-didehydrojasmonate (J7), an analogue of methyl jasmonate, on the *in vitro* growth of human cervical carcinoma HeLa cells. Significantly decreased rates of viability ($IC_{50} \sim 15 \mu M$) as well as evidence of apoptosis were observed with J7. Cell morphological changes observed under light microscopy confirmed apoptosis occurrence. Furthermore, the results from Annexin V-FITC/PI double staining and the cell cycle arrest assay indicated that J7 induced earlier apoptosis of HeLa cells. J7 also reduced the expression of Bcl-2 and subsequent activation of a protease cascade involving caspase-9 and -3 by Western blot assay was observed. We also found that J7 was able to induce DNA damage. These findings suggest that J7 induces HeLa cell apoptosis by activation of caspase pathway and the apoptotic effect is associated with DNA damage. Therefore, J7 may be a candidate compound to be developed into an anticancer agent.

Introduction

Methyl jasmonate is a member of the plant stress hormone family of jasmonates. It has been reported that methyl jasmonate inhibits cell proliferation and induces cell death in various human and mouse cancer cell lines, such as breast, prostate, lung, melanoma, lymphoblastic leukemia and lymphoma cells (1-3). Methyl jasmonate exhibited selective cytotoxicity to transformed cells while sparing normal blood lymphocytes, even when the latter were part of a mixed population of leukemic and normal cells drawn from the blood of chronic lymphocytic leukemia (CLL) patients (4,5). Recently methyl 5-chloro-4,5-didehydrojasmonate (J7) (Fig. 1),

a new analogue of methyl jasmonate was synthesized and evaluated for its inhibitory effects on the production of pro-inflammatory mediators (NO, IL-6 and TNF- α) in lipopolysaccharide (LPS)-activated RAW264.7 murine macrophage cells (6). However, the biological properties of J7 with regard to anti-proliferative effects and to molecular mechanisms of the therapeutic effects have not been examined.

In this study, we examined the anticancer activity of J7 on human cervical carcinoma HeLa cells. Many reports have demonstrated that exposure of cancer cells to chemotherapeutic drug can lead to DNA damage (7,8), and the most DNA lesion produced is the DNA strand breakage, which can be sensitively detected by the alkaline microgel electrophoresis (comet) assay. The comet assay assesses DNA damage from individual cells based on the migration of denatured DNA through an electrophoretic field. Many studies have used the comet assay to investigate DNA damage in a wide range of tumor cells in response to a variety of DNA-damaging agents (9,10). Cells with damaged DNA can undergo cell cycle arrest, facilitate DNA repair or undergo apoptosis.

Apoptosis has been characterized as a homeostatic mechanism to maintain cell populations in tissues and also occurs as defense mechanism such as in immune reactions. It plays a protective role against carcinogenesis by eliminating either damaged cells or those excess abnormal cells that proliferated due to induction by various chemical agents (11,12). The characteristics of apoptotic death include cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and the formation of plasma membrane-bound apoptotic bodies (13). Recent experiments have indicated that mitochondria play an essential role in apoptosis commitment (14). In response to apoptotic stimuli, several important events occur at the mitochondria. These apoptotic mitochondrial events can be controlled and regulated by members of the Bcl-2 family of proteins (15). Bcl-2 is an anti-apoptotic protein and its main mechanism of action is the suppression of cytochrome *c* release from the mitochondria via inhibition of mitochondrial permeability transition. Once Bcl-2 expression is reduced, cytochrome *c* is released from the mitochondria into cytosol and interacts with Apaf-1 (apoptotic protease activation factor 1), leading to the activation of procaspase-9. Active caspase-9 then

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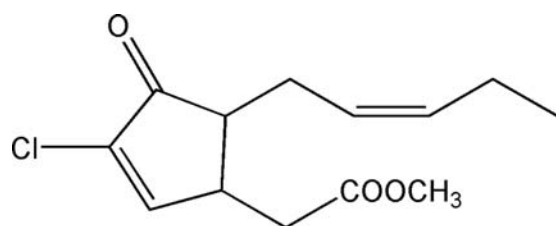


Figure 1. The structure of methyl-5-chloro-4,5-didehydrojasmonate (J7).

activates caspase-3, which subsequently activates the rest of the caspase cascade and leads to apoptosis (16).

Emerging evidence has demonstrated that induction of apoptosis is a current preferred way to treat cancer (17,18). In our study, we explored the anticancer activity of J7 expecting to identify a novel apoptosis-inducing compound that can be a candidate antitumor agent. J7 is a small molecule and can be easily synthesized and structurally manipulated for selective development. In addition, methyl jasmonate, the starting material for the synthesis of J7, is widespread in plant and some lower eukaryotes. Therefore, J7 has a great potential to be developed into an anticancer drug. Meanwhile, the clarification of the molecular mechanisms of action of J7 is also important in developing its applications.

Materials and methods

Chemicals. PI/RNase staining buffer for cell cycle analysis and Annexin-FITC kit for apoptosis were from BD Biosciences Pharmingen, USA. Dimethyl sulfoxide (DMSO) and PBS (pH 7.4) were purchased from Sigma Chemical Co. Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Hyclone Laboratories (Logan, UT). CCK-8 was purchased from Dojin Laboratories (Osaka, Japan). All other chemicals were of analytical reagent grade.

Preparation of J7. J7 was synthesized from methyl jasmonate and identified according to the spectrometric data: ^1H NMR (CDCl_3 , 400 MHz): δ 7.52 (1H, d, $J=2.4$ Hz, H-4), 5.48 (1H, m, H-10), 5.21 (1H, m, H-9), 3.70 (3H, s, OCH_3), 2.96 (1H, m, H-3), 2.60 (1H, dd, $J=16.0, 6.4$ Hz, H-2b), 2.56-2.51 (1H, m, H-8b), 2.45 (1H, dd, $J=16.0, 7.6$ Hz, H-2a), 2.35 (1H, m, H-8a), 2.22 (1H, m, H-7), 2.04 (2H, m, H-11), 0.94 (3H, t, $J=7.6$ Hz, H-12); ^{13}C NMR (CDCl_3 , 100 MHz): δ 201.4 (C-6), 171.4 (C-1), 158.1 (C-4), 135.7 (C-5), 135.2 (C-10), 123.6 (C-9), 52.0 (OCH_3), 50.2 (C-7), 40.4 (C-3), 37.9 (C-2), 27.8 (C-8), 20.6 (C-11), 14.1 (C-12); FABMS m/z 257 (M + H) $^+$; HRFABMS m/z 257.0937 (M + H) $^+$. Stock solutions of J7 were prepared in DMSO and kept at 4°C. Further dilutions were made immediately prior to each experiment.

Cell lines. HeLa cells obtained from American Type Culture Collection (ATCC) were cultured in EMEM medium supplemented with 10% fetal bovine serum at 37°C (5% CO_2) in a humidified atmosphere.

Cell viability assay. HeLa cells were plated at 5×10^3 cells into each well of a 96-well microplate. After the cells were

cultured for 24 h, J7 at various concentrations was added to each well as treatment and an appropriate volume of drug vehicle (DMSO) was used as the control. The plate was incubated further for 48 h. Then, 10 μl of CCK-8 reagent was added and incubated for a further 2 h. The cell viability was assessed by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium], an indicator that is reduced by dehydrogenases in cells to give a yellow-colored product (formazan), which is soluble in cell culture medium. The optical density for living cells was read at 450 nm in a multi-microplate reader (synergy HT, Bio-Tek®) (19).

DNA content analysis. The cells (3×10^5 cells in 60 mm dish) treated with or without J7 were collected by trypsinization and washed with cold PBS via centrifugation. The cells were suspended in PBS, fixed with 70% ethanol (v/v). After overnight incubation, samples were washed with cold PBS and stained with PI/RNase staining buffer (BD Pharmingen) for 15 min at room temperature. The cells in different phases of the cell cycle were analyzed using a FACScan flow cytometer analysis system (Becton-Dickinson, San Jose, CA), and 20,000 events were analyzed for each sample. The percentage of cells in the different phases of cell cycle was determined using Modfit software (Becton-Dickinson Instruments). All experiments were performed in triplicate and yielded similar results.

Measurement of apoptotic cell morphology. HeLa cells were distributed (1×10^5 cells/well) into a 24-well plate and allowed to adhere overnight. The cells were treated with J7 (10 and 15 μM) for 48 h. Non-treated wells received an equivalent volume of DMSO (<0.1%). Optic phase-contrast photographs were taken with a Nikon™ Phase Contrast-2, ELWD 0.3 inverted microscope. Each experiment was repeated at least three times.

Annexin V-FITC/PI apoptotic analysis. The cells (3×10^5 cells in 60 mm dish) treated with or without J7 were collected by trypsinization and washed with cold PBS via centrifugation. Then, 1×10^5 cells were resuspended in 100 μl of binding buffer and stained with 5 μl of Annexin V/FITC (BD Pharmingen) and 10 μl of PI (50 $\mu\text{g}/\text{ml}$) for 15 min at room temperature in the dark. The samples were then read immediately in a FACS caliber flow cytometer. Ten thousand events were recorded in each analysis. Analyses were performed by the CellQuest software (Becton-Dickinson Instruments, Franklin Lakes, NJ).

Western blot assay. The cells (1×10^6 cells in 100 mm dish) treated with or without J7 were harvested and washed with cold PBS. Cell pellets were lysed in protein extraction solution (PRO-PREP) for 20 min on ice. Lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Protein contents in the supernatant were measured using the Bradford protein assay (Bio-Rad, Hercules, CA). Total cellular protein (50 μg) was separated on 10 and 14% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Schleicher & Schuell, USA). The membranes were blocked with 5% BSA or 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 and then incubated with primary antibodies (Cell Signaling

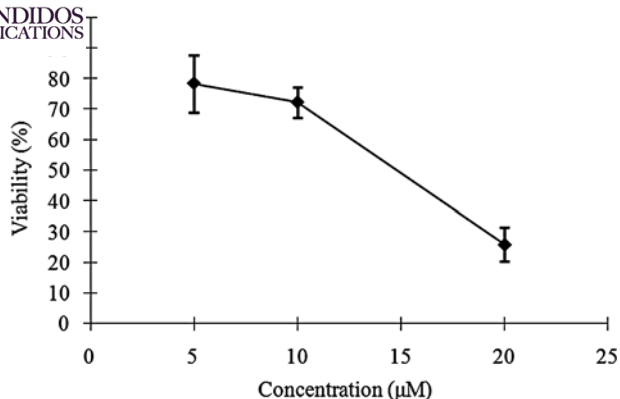


Figure 2. Effect of J7 on the viability of HeLa cells. HeLa cells were treated with various concentrations of J7 for 48 h. Relative cell viability was determined by WST-8 and is presented as the percentage of control cells. The data are shown as means \pm SD of three independent experiments.

or Santa Cruz). The primary antibodies were then stained with horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected using an enhanced chemiluminescence detection system according to the manufacturer's manual (Amersham Pharmacia Biotech, Amersham, UK).

Single-cell gel electrophoresis. To examine the DNA damage in these single-cell suspensions, we performed an alkaline single-cell gel electrophoresis (comet assay). The comet assay was carried out based on the method proposed by Singh (20) with some modifications. All steps were carried out under indirect light. Briefly, the cells (treated with or without J7 for 24 h) were pelleted and resuspended in 0.5% low melting point agarose at 37°C and layered on a frosted microscope slide previously coated with a thin layer of 1% normal melting agarose and kept for 10 min on ice. After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100 and 10% DMSO for 1 h 30 min at 4°C). The slides were then incubated in an alkaline buffer (0.3 M NaOH, 1 mM Na₂-EDTA) for 40 min at 4°C. Electrophoresis was carried out for 40 min at 25 V. After electrophoresis, the slides

were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (2 µg/ml) and observed under fluorescent microscopy (Leica DM LB2).

The data were analyzed using the Komet 5.5 software. Olive Tail Moment (OTM) was used to evaluate DNA damage. OTM is defined as the percentage of DNA in the comet tail multiplied by the distance between the means of the head and tail distributions (21). Olive Tail Moment = (Tail mean - Head mean) \times Tail% DNA/100.

Statistical analysis. Each experiment was repeated at least three times and the results were expressed as mean \pm SEM in some quantitative experiments. All the results reported were obtained from at least three independent experiments with similar results.

Results

J7 inhibits HeLa cell growth and induces apoptotic cell death. To explore the cytotoxic effect of J7, cytotoxicity assay was performed to determine the IC₅₀ value in HeLa cells. The water-soluble tetrazolium salt WST-8 was used as an indicator in the cytotoxicity assay (19). As shown in Fig. 2, treatment of HeLa cells with various concentrations of J7 caused a concentration-dependent decrease in cell number, with an IC₅₀ \sim 15 µM. With a higher concentration (up to 40 µM) of J7 the cells were all killed (data not shown).

To investigate whether the J7-induced HeLa cell death is associated with apoptosis or cell cycle arrest, treated or untreated HeLa cells with J7 were stained with PI and measured using a flow cytometry. As shown in Fig. 3, after treatment with 10 and 20 µM of J7 for 48 h, the percentage of cells in G2 phase significantly increased from a control value of 12.35-14.57 and 26.44%, respectively, and the accumulation of cells in the G2/M phase was accompanied by synchronous decreases in the percentage of cells in the G1 phase. We also observed the appearance of sub-G1 peak corresponding to the population of cells with apoptotic DNA content. After treated with 10 µM of J7 for 48 h, the population of apoptotic cells significantly increased from a control value of 4.82-13.60%. However, the proportion of apoptotic cells treated with 20 µM of J7 slightly increased compared with control.

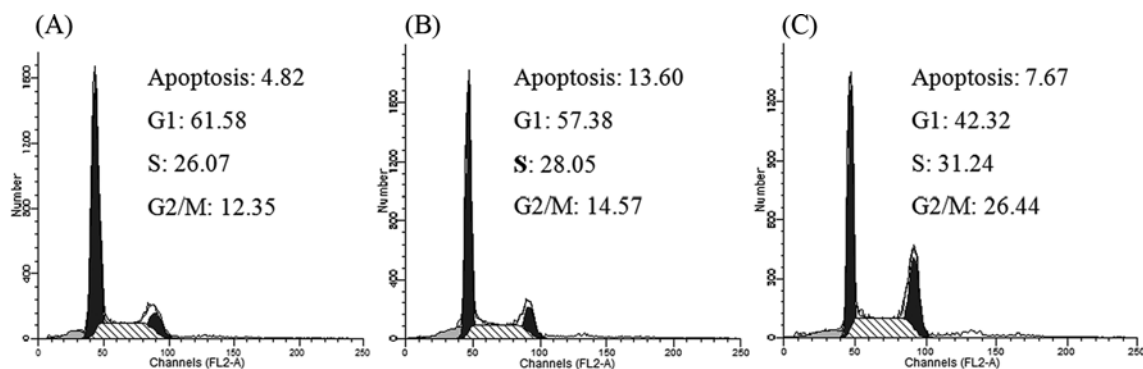


Figure 3. Effect of J7 on cell cycle progression of HeLa cells. HeLa cells were treated with vehicle alone (A) or with 10 or 20 µM of J7 for 48 h (B) and (C). DNA content was analyzed by flow cytometry with PI staining. The cell cycle distribution was calculated as the percentage of cells containing apoptosis, G0/G1, S and G2/M phase. Data are representative of three independent experiments.

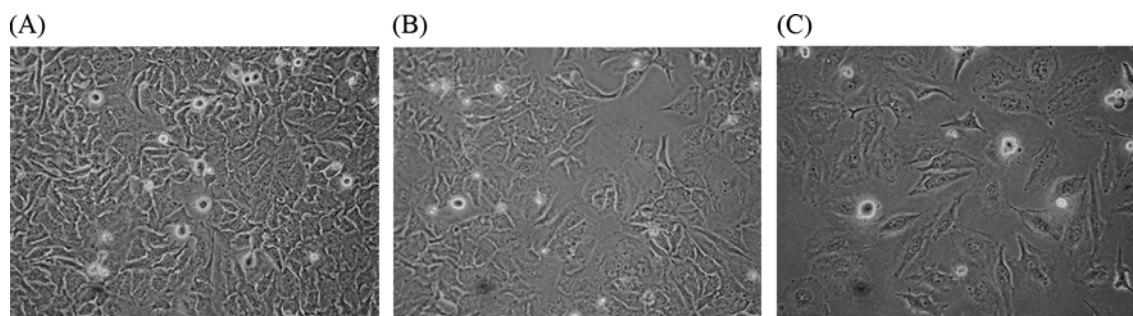


Figure 4. Morphological changes in HeLa cells were observed under light microscopy. Exponentially growing cells were incubated either with vehicle alone (A) or 10 μ M (B) or 15 μ M (C) of J7 for 48 h. Magnification, \times 160.

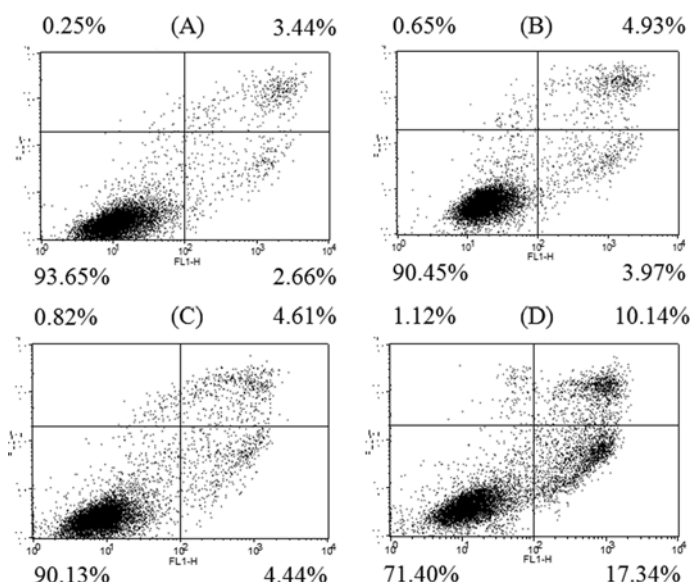


Figure 5. Measurement of apoptotic population induced by J7. HeLa cells were treated with vehicle alone for 24 (A) and 48 h (C), 15 μ M of J7 for 24 (B) and 48 h (D). Cells were harvested at the indicated time-points, and stained with Annexin V-FITC/PI and analyzed for apoptosis by flow cytometry. The cell populations shown in the lower left represent living cells, lower right represents earlier apoptosis cells, upper right represents late apoptosis or necrosis cells and upper left represents pre-necrotic cells. All experiments were performed in triplicate and gave similar results.

We focused on the apoptosis of HeLa cells induced by J7 due to our research interest. Thus, morphological observation was first implemented. Morphological changes in the cells treated with various concentrations of J7 using light microscopy revealed apoptosis occurrence. Under light microscopy, non-treated HeLa cells spread regularly in the culture plates and grew to near confluence (Fig. 4A). After treatment with 10 μ M of J7 for 48 h, some HeLa cells floated, but the major attached cells kept a normal cellular shape (Fig. 4B). After treatment with IC₅₀ value of J7 for 48 h, a significant proportion of the HeLa cells detached from the plate and most of the remaining attached cells showed typical morphological features of apoptosis such as cellular shrinkage and disruption (Fig. 4C). Some floating cells were observed in the control HeLa cells.

To obtain further evidence for the induction of apoptosis by J7 in HeLa cells, Annexin V and PI double staining assay was performed. Significant differences were observed between the untreated and treated cells. As shown in Fig. 5A, 93.65% of HeLa cells treated with vehicle alone were viable (Annexin V⁻PI⁻), and ~2.66% were early apoptotic cells

(Annexin V⁺PI⁻). After treatment with 15 μ M of J7 for 24 h, only 3.97% of HeLa cells were apoptotic (Fig. 5B). But after 48 h treatment, the proportion of apoptotic cells increased to 17.34% (Fig. 5D). In addition, the percentage of late apoptotic cells (or necrosis cells) stained both by Annexin V-FITC and PI (Annexin V⁺PI⁺) was also increased by treatment of J7. Therefore, in comparison with control cells, J7-treated HeLa cells displayed an increase in apoptosis.

J7-induced apoptosis is associated with the activation of a protease cascade involving caspase-9 and -3. Caspases play a central role in apoptosis. Once activated, caspases can activate other procaspases, allowing initiation of a protease cascade. This proteolytic cascade amplifies the apoptotic signaling pathway and thus leads to rapid cell death (22). In order to characterize the molecular mechanism of J7-induced apoptosis in HeLa cells, we evaluated whether caspase-dependent signal pathways were involved in the apoptotic cell death induced by J7 in HeLa cells. HeLa cells were treated with 10 and 15 μ M of J7 (according to the data of cytotoxicity and

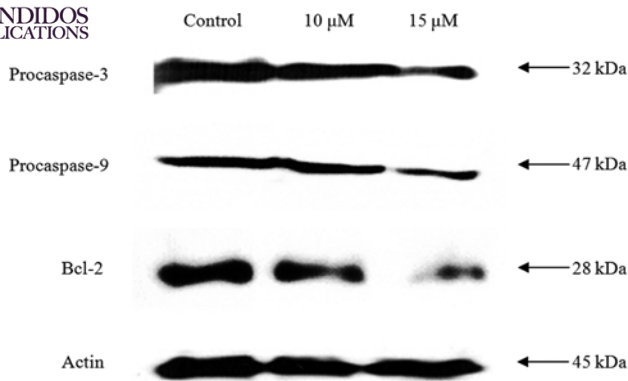


Figure 6. Involvement of protein activation in J7-induced apoptosis. HeLa cells were treated with 10 and 15 μM of J7 for 48 h. Extracts from untreated or J7-treated cells were assayed by Western blot analysis.

DNA content analysis) for 48 h. Western blot assay exhibited dramatic decreases in procaspase-3 and -9 protein level (Fig. 6). To explore whether this apoptotic event induced by J7 is mitochondria-dependent, Bcl-2 which is involved in governing mitochondrial membrane permeability was measured after treatment with J7. As shown in Fig. 6, Bcl-2 protein level significantly decreased in a concentration-dependent manner, suggesting that the J7-induced apoptosis

of HeLa cells was via caspase-dependent mitochondrial pathway.

J7-induced apoptosis is associated with induction of DNA damage. Since above results exhibited apoptotic cell death in HeLa cells, we hypothesized that J7 could induce DNA damage that might contribute to the apoptosis in HeLa cells. To test this hypothesis, we performed an alkaline single-cell gel electrophoresis (comet assay), a sensitive method that evaluates DNA single-strand breaks at the single-cell level based on the increased tail of DNA migration. Fig. 7B shows the DNA migration patterns in HeLa cells. When HeLa cells were treated with 15 μM of J7 for 24 h, DNA damage started to be evident, as indicated by the increased tail of DNA migration (Fig. 7B) and the Olive Tail Moment data (Fig. 7A). After treatment with 30 μM of J7, almost the entire population of HeLa cells contained damaged DNA. At the same time after 24 h treatment, the apoptosis was barely detectable (Fig. 5). Thus, J7 induces DNA single strand breaks before apoptosis induction.

Discussion

Our result indicated that J7 showed cytotoxicity on HeLa cells and its IC_{50} value was $\sim 15 \mu\text{M}$ after 48 h treatment. Compared with J7, the IC_{50} value of methyl jasmonate was

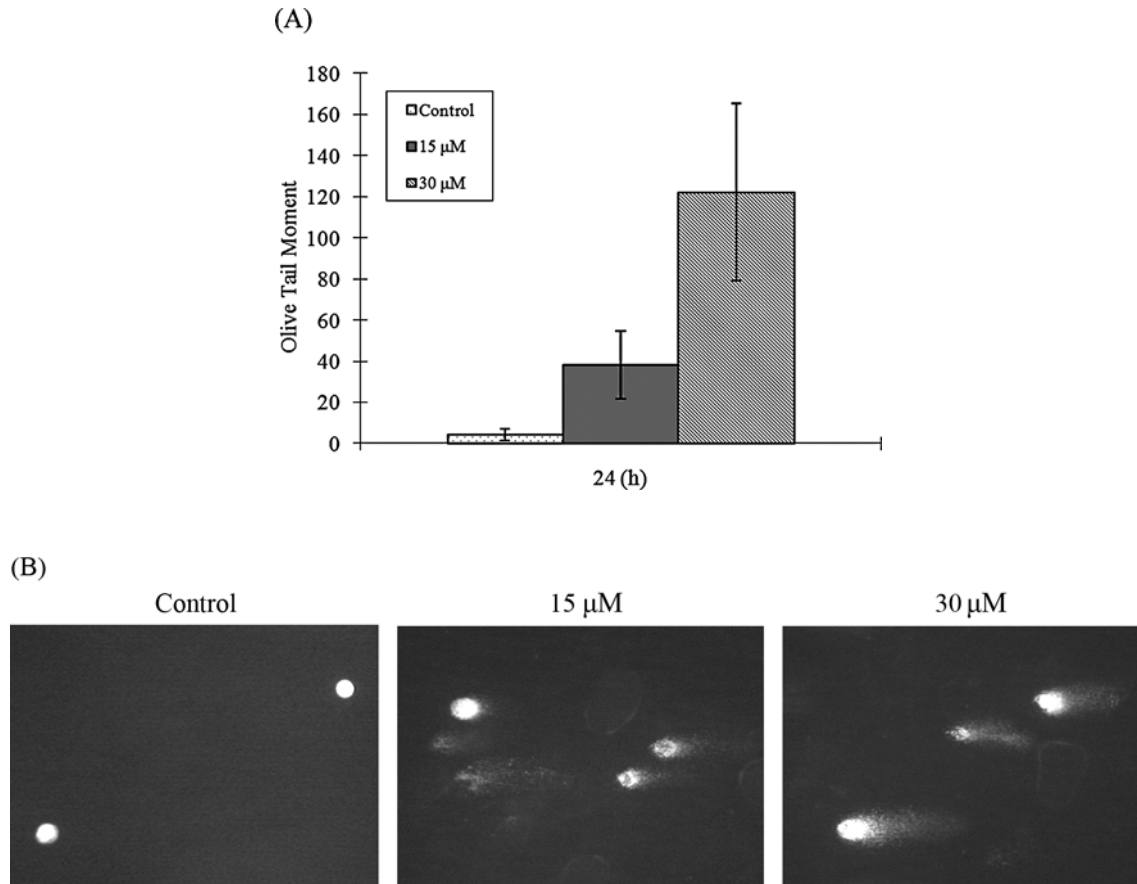


Figure 7. J7 induces DNA strand breaks in HeLa cells. (A) Olive Tail Moment of DNA from HeLa cells treated with 15 and 30 μM of J7. (B) DNA single-strand breaks induced by J7 in HeLa cells. Cells were incubated with or without 15 and 30 μM of J7 for 24 h. The comet assay was determined as described in Materials and methods. Results are from three independent experiments.

3,000 μM for HeLa cells after 24 h treatment (23). Although longer incubation with methyl jasmonate reduces the IC_{50} value, we suggest that J7 has much greater cytotoxic effect on HeLa cells than methyl jasmonate. Thus, J7 can be considered as a new promising lead compound for cancer treatment in human.

Chemotherapeutic agents exert their anticancer activity through blocking cell cycle progression and triggering tumor cell apoptosis. In order to ensure the mode of action of J7, DNA content analysis was carried out using flow cytometry. After 48 h treatment, we observed great sub-G1 peak and G2 phase arrest in 10 and 20 μM of J7 treated HeLa cells, respectively (Fig. 3). This result indicated that J7-induced HeLa cell death could be associated with both apoptosis and cell cycle arrest. It should be noted that after 20 μM of J7 treatment, the percentage of sub-G1 peak significantly decreased compared with 10 μM of J7 treated cells, indicating that less apoptotic cell death was induced under high concentration of J7. Therefore, concentrations lower than 20 μM of J7 were used in the following series of assays which focused on apoptosis due to our research interest.

A variety of methods have been reported for monitoring different steps in apoptotic pathways (22). The initiating effect of J7 on the HeLa cell apoptosis was confirmed by observing the cytomorphological alterations under light microscopy. The predominant pattern of the abnormal metaphase morphology in the cells exposed to J7 suggested a pronounced and specific impairment with typical apoptotic patterns including a reduction in the volume and fragmentation of cells (Fig. 4). The result of the Annexin V-FITC/PI double staining assay further indicated that J7 induced early apoptosis in HeLa cells at 48 h (Fig. 5). On the basis of these data, we conclude that J7 exhibits its anti-proliferation and cytotoxicity effects on HeLa cells partly by triggering apoptosis in the cells.

To gain clear insight into the mechanisms of J7-induced apoptosis, the expression levels and activities of the proteins involved in apoptosis were detected by Western blot assay. In mammalian cells, apoptosis pathway can be divided into caspase-dependent and -independent pathways (24,25). Caspases involved in apoptosis can be grouped into 'apoptotic initiator', such as caspase-9, and 'apoptotic effector', such as caspase-3 based on the substrate specificities and target proteins of caspases (22). In our study, significant decreases of procaspase-3 and -9 levels were observed, which demonstrated the activation of caspase-dependent apoptosis pathway. The activation of this pathway is initiated by mitochondrial released cytochrome *c* which combines with Apaf-1, procaspase-9 and ATP in the cytosol, producing active caspase-9 (16). The release of cytochrome *c* from mitochondria can be suppressed by overexpression of Bcl-2. In our study, J7-treated cells showed much lower level of Bcl-2 compared with untreated cells (Fig. 6). The decrease of Bcl-2 leads to change of the mitochondrial permeability, promoting cytochrome *c* release which then initiates caspase-dependent pathway. It has been shown that Bcl-2 overexpression plays a key role in tumors resistant to chemotherapeutic drugs (26,27) and radiotherapy (28,29). Therefore, J7 may have a great potential to be combined with other anticancer agents and radiation therapy to increase tumor sensitivity to drugs and radiation.

Apoptosis can be triggered by multiple mechanisms, including DNA damage (30,31). The cell's alternative to repairing damaged cells can be simply to eliminate them through the process of apoptosis. Results from comet assay showed that nearly 100% of the cell population contained DNA strand breaks after 24 h of incubation with J7 (Fig. 7B). However, no increase of earlier apoptotic cells as compared to control after 24 h treatment was noted (Fig. 5), suggesting that the apoptosis-associated DNA fragmentation had not yet occurred. While our data strongly suggest that J7 has the ability to induce DNA strand breaks, the mechanism of breakage and the signal proteins linking DNA damage to apoptosis remain elusive. In addition, whether J7-induced apoptosis is associated with G2 phase arrest (Fig. 3) should be further investigated.

In conclusion, our study provides experimental evidence that J7-induced apoptosis of HeLa cells was accompanied with cellular shrinkage and disruption. J7 induces apoptosis in HeLa cells by reduction of Bcl-2 expression, which promotes cytochrome *c* release followed by activation of caspase-9 and -3. J7-induced DNA damage is beyond the capacity of DNA repair system. Thus, cells execute apoptosis to eliminate the damaged cells.

To the best of our knowledge, there are no reports on anticancer activity of J7, and the present study serves as the first attempt to evaluate the action of methyl 5-chloro-4,5-didehydrojasmonate on HeLa cells. Although the target molecules of J7 action should be further investigated, our current studies indicate that J7 has great potential as a new lead compound that could be developed into a novel anti-cancer agent.

Acknowledgements

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