

Suppression of U937 human monocytic leukemia cell growth by dideoxypetrosynol A, a polyacetylene from the sponge *Petrosia* sp., via induction of Cdk inhibitor p16 and down-regulation of pRB phosphorylation

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Abstract. Dideoxypetrosynol A, a polyacetylene from the marine sponge *Petrosia* sp., is known to exhibit significant selective cytotoxic activity against a small panel of human tumor cell lines, the mechanisms of which however, are poorly understood. The aim of the present study was to further elucidate the possible mechanisms by which dideoxypetrosynol A exerts its anti-proliferative action in cultured human monocytic leukemia U937 cells. We observed that the proliferation-inhibitory effect of dideoxypetrosynol A was due to the induction of G1 arrest in the cell cycle, the effects of which were associated with up-regulation of cyclin D1 and down-regulation of cyclin E, in a concentration-dependent manner without any change in cyclin-dependent-kinases (Cdks) expression. Dideoxypetrosynol A markedly induced the levels of Cdk inhibitor p16/INK4a expression. Furthermore, down-regulation of phosphorylation of retinoblastoma protein (pRB) by this compound was associated with enhanced binding of pRB and transcription factor E2F-1. Overall, our results demonstrate a combined mechanism involving the inhibition of pRB phosphorylation and induction of p16 as targets for dideoxypetrosynol A, may explain some of its anti-cancer effects.

Introduction

The mammalian cell cycle is orchestrated by the sequential activation-inactivation of a series of cyclin-dependent kinases (Cdks) and their inhibitor proteins (1,2). D-type cyclins and cyclin E are required for progression through G1. As cells enter G1, the cyclin D/Cdk4 (and/or Cdk6) complex appears to be necessary for transition through early G1, whereas the cyclin E/Cdk2 complex is required in transition from late G1 into S phase (1,3,4). Cyclin A is produced in late G1 and its expression accumulates during S and G2 phase, whereas expression of B-type cyclins is typically maximal during G2 to M phase transition and it controls the passage through M phase. Cyclin A primarily associates with and activates Cdk2, whereas B-type cyclins are the major activator of Cdk2 (also known as Cdk1) (5). Cdk inhibitors are divided into two families according to substrate specificity. In mammalian cells, these are the CIP/KIP family, consisting of p21, p27 and p57, and the INK4a family, including p15, p16 and p18 (6). Cdk inhibitors mediate cell cycle arrest in response to several antiproliferative signals. The activity of Cdks is also negatively regulated by binding to Cdk inhibitors in response to a variety of antiproliferative signals and thus modulates retinoblastoma protein (pRB) phosphorylation events, which are essential for various cell cycle transitions (7,8). These observations suggest new approaches that could alter uncontrolled human cancer cell growth by modulating cell cycle regulators causing cell cycle arrest and could be useful in the prevention and/or intervention of human cancer(9).

Recent studies indicated that the marine environment has proven to be a very rich source of bioactive compounds (10,11). Marine sponges, a kind of sedentary marine species, have been used as a main source for this study of hitherto unknown biological activities of natural marine products. Components of marine sponges are known to modulate various

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Table I. Gene-specific primers for RT-PCR.

Name	Sequence of primers
Cyclin D1	
sense	5'-TGG-ATG-CTG-GAG-GTC-TGC-GAG-GAA-3'
antisense	5'-GGC-TTC-GAT-CTG-CTC-CTG-GCA-GGC-3'
Cyclin E	
sense	5'-AGT-TCT-CGG-CTC-GCT-CCA-GGA-AGA-3'
antisense	5'-TCT-TGT-GTC-GCC-ATA-TAC-CGG-TCA-3'
Cdk2	
sense	5'-GCT-TTC-TGC-CAT-TCT-CAT-CG-3'
antisense	5'-GTC-CCC-AGA-GTC-CGA-AAG-AT-3'
Cdk4	
sense	5'-ACG GGT GTA AGT GCC ATC TG-3'
antisense	5'-TGG TGT CGG TGC CTA TGG GA-3'
Cdk6	
sense	5'-CGA ATG CGT GGC GGA GAT C-3'
antisense	5'-CCA CTG AGG TTA GAG CCA TC-3'
p16	
sense	5'-CGG-AAG-GTC-CCT-CAG-ACA-TC-3'
antisense	5'-TCA-TGA-AGT-CGA-CAG-CTT-CCG-3'
p21	
sense	5'-CTC-AGA-GGA-GGC-GCC-ATG-3'
antisense	5'-GGG-CGG-ATT-AGG-GCT-TCC-3'
p27	
sense	5'-AAG-CAC-TGC-CGG-GAT-ATG-GA-3'
antisense	5'-AAC-CCA-GCC-TGA-TTG-TCT-GAC-3'
GAPDH	
sence	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
antisense	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

biological activities such as anti-tumor, anti-inflammatory or immunomodulatory effects (12-14). Through the screening of natural compounds that induce cell cycle arrest and/or apoptosis, we previously reported that dideoxypetrosynol A, a polyacetylene from the sponge *Petrosia* sp., has significant selective cytotoxic activity against a small panel of human solid tumor cell lines by inhibiting DNA replication and apoptotic cell death (14-16). However, the molecular mechanisms of its anti-proliferative action on malignant cell growth are not completely known. The present study was carried out to characterize the probable mechanisms involved in dideoxypetrosynol A-mediated growth inhibitory effect in human monocytic leukemia U937 cells. We demonstrated that dideoxypetrosynol A induced cell cycle arrest at G1 phase through a combined mechanism involving the induction of Cdk inhibitor p16 and down-regulation of the phosphorylation of pRB.

Materials and methods

Cell culture, dideoxypetrosynol A and cell proliferation study. The human leukemia cell line, U937, was purchased from the American Type Culture Collection (Rockville, MD),

and maintained at 37°C in humidified conditions of 95% air and 5% CO₂ in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Dideoxypetrosynol A (Fig. 1A) was prepared as described previously (15) and dissolved in dimethyl sulfoxide as a stock solution at 10 mg/ml concentration, and stored in aliquots at -20°C. Measurement of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. For the morphological study, cells were treated with dideoxypetrosynol A for 48 h and directly photographed with an inverted microscope.

Flow cytometric analysis. After treatment with dideoxypetrosynol A, cells were collected, washed with cold PBS, and fixed in 75% ethanol at 4°C for 30 min. DNA contents of cells were measured using a DNA staining kit (CycleTEST™ PLUS kit, Becton-Dickinson, San Jose, CA). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit protocol. Fluorescence intensity was determined using a

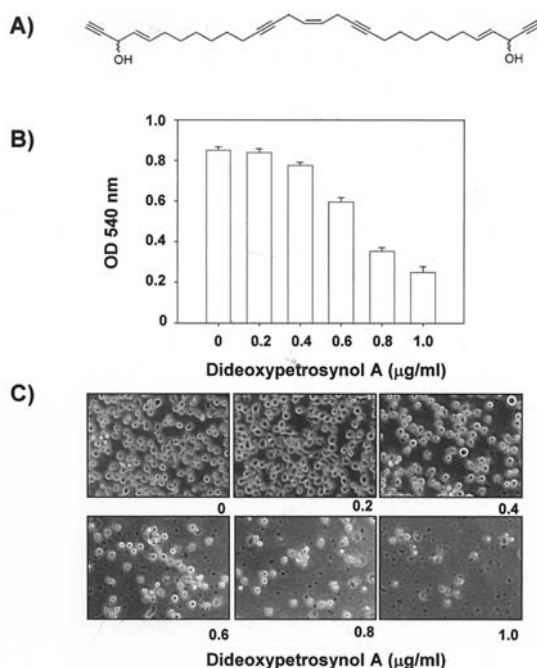


Figure 1. Growth inhibition and morphological changes by dideoxypetrosynol A treatment in U937 cells. (A) Chemical structure of dideoxypetrosynol A. (B) Cells were plated at 4×10^4 cells per 60-mm plate, and incubated for 24 h. The cells were treated with variable concentrations of dideoxypetrosynol A for 48 h and growth inhibition was measured by the metabolic-dye-based MTT assay. Results are expressed as the means \pm S.E. of three independent experiments. (C) Cells were incubated with variable concentrations of dideoxypetrosynol A. After incubation for 48 h, the cells were examined under light microscopy. Magnification $\times 200$.

FACScan flow cytometer and analyzed by CellQuest software (Becton-Dickinson).

RNA extraction and reverse transcription-PCR. Total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA) and primed with random hexamers to synthesize complementary DNA using AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out in a Mastercycler (Eppendorf, Hamburg, Germany) with indicated primers in Table I. The conditions for PCR reactions were $1 \times 94^\circ\text{C}$ for 3 min, $35 \times 94^\circ\text{C}$ for 45 sec, 58°C for 45 sec, and 72°C for 1 min; and $1 \times 72^\circ\text{C}$ for 10 min. Amplification products obtained by PCR were electrophoretically separated on 1% agarose gel and visualized by ethidium bromide (EtBr) staining.

Immunoprecipitation, gel electrophoresis and Western blot analysis. The cells were harvested, lysed, and protein concentrations were quantified using the BioRad protein assay (BioRad Lab., Hercules, CA), following the procedure described by the manufacturer. For immunoprecipitation, cell extracts were incubated with an immunoprecipitating antibody in extraction buffer for 1 h at 4°C . The immuno-complex was collected on protein G/A-Sepharose beads (Sigma). Western blot analysis was performed as described (17). Briefly, the immunoprecipitated or an equal amount of total protein was subjected to electrophoresis on SDS-polyacrylamide gels and

Table II. Effects of dideoxypetrosynol A on the distribution of the cell cycle in U937 cells.

dideoxy- petrosynol A (μg/ml)	% of cell		
	G1	S	G2/M
0	46.29	28.74	24.97
0.2	53.89	25.07	21.04
0.4	57.07	23.02	19.91
0.6	60.37	22.55	17.08
0.8	63.84	20.24	15.92

Cells have been grown in different concentrations of dideoxypetrosynol A for 48 h, and then analyzed by flow cytometry as described in Materials and methods. Data are presented as the mean values obtained from two independent experiments.

transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electroblotting. Blots were probed with the desired antibodies for 1 h, incubated with diluted enzyme-linked secondary antibody and then visualized using enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp.). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Results

Growth inhibition and G1 arrest by dideoxypetrosynol A. To evaluate the effects of dideoxypetrosynol A on cell proliferation, we initially determined the effects of dideoxypetrosynol A on the growth of U937 cells. For this purpose, cells were treated with dideoxypetrosynol A (0.2 – $1.0 \mu\text{g/ml}$) for 48 h and viable cells were measured by MTT assay. As shown in Fig. 1, dideoxypetrosynol A had a strong inhibitory effect on cell proliferation in a dose-dependent manner, which was associated with a distinct morphological change including membrane ruffling. To determine whether dideoxypetrosynol A treatment of cells resulted in the alteration of cell cycle progression, the cell cycle patterns of the U937 cells were examined. Analysis of the cell cycle distribution of cells after exposure to dideoxypetrosynol A showed that these cells had a marked accumulation in the G1 phase of the cell cycle (Table II). This was accompanied by a decrease in their S and G2/M phases when compared with the untreated control cells, which suggests that the growth inhibitory effect of dideoxypetrosynol A in U937 cells was the result of a block during this G1 phase.

Effects of dideoxypetrosynol A on the levels of G1 phase cell cycle regulators. Since dideoxypetrosynol A arrested U937 cells in the G1 phase of the cell cycle, we determined the expression levels of the cell cycle regulating factors at the G1 boundary, such as cyclin D1, cyclin E, Cdk2, Cdk4 and Cdk6,

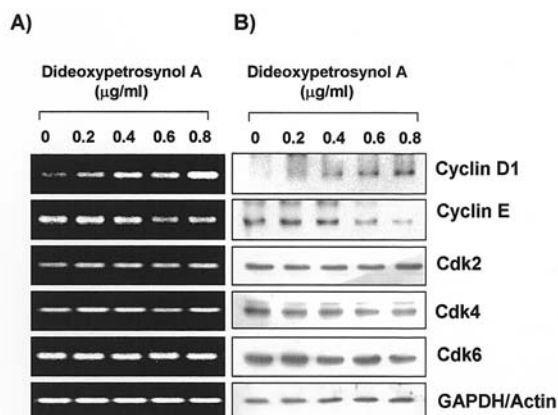


Figure 2. Effects of dideoxypetrosynol A on the protein levels of cyclins and Cdk in U937 cells. (A) After 48-h incubation with dideoxypetrosynol A, total RNAs were isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with indicated primers and the reaction products were subjected to electrophoresis in a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) The cells were lysed and then cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

by RT-PCR and Western blotting. As shown in Fig. 2, the protein and mRNA levels of cyclin D1 were significantly increased by dideoxypetrosynol A treatment and the levels of cyclin E were decreased in a concentration-dependent manner. However, the levels of Cdk (Cdk2, Cdk4 and Cdk6) remained unchanged in dideoxypetrosynol A-treated cells. These results suggest that the suppressive effects of dideoxypetrosynol A at the G1 phase in U937 cells are partly caused by up-regulating the levels of cyclin D1 and down-regulating the levels of cyclin E.

Induction of Cdk inhibitor p16/INK4a by dideoxypetrosynol A. To further understand the anti-proliferative mechanism of dideoxypetrosynol A, we next investigated whether Cdk inhibitors, such as p16/INK4a, p21/WAF1/CIP1 and p27/KIP1, are involved in the dideoxypetrosynol A-induced growth arrest in U937 cells (Fig. 3). In the untreated control cells, the protein and mRNA levels of p16 were undetectable. However, the incubation of cells with dideoxypetrosynol A caused a striking concentration-dependent increase in the induction of p16 protein and mRNA, whereas the compound did not significantly affect the expression levels of other Cdk inhibitors, including p21 and p27. The data suggested that the dideoxypetrosynol A-induced G1 cell cycle arrest in U937 cells requires increased p16 expression, but not p21 and p27 expression.

Down-regulation of pRB phosphorylation and increased binding of pRB and E2F-1 by dideoxypetrosynol A. Since the RB gene product pRB is an important checkpoint protein in the G1 phase of the cell cycle, we next determined the kinetics between phosphorylation of pRB and transcription factor E2F-1. The total levels of pRB expression were decreased remarkably and changed from a hyperphosphorylated form to a hypophosphorylated form by dideoxypetrosynol

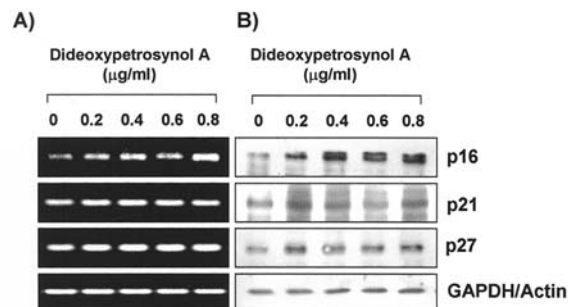


Figure 3. Induction of Cdk inhibitor p16 by dideoxypetrosynol A treatment in U937 cells. (A) After 48-h incubation with dideoxypetrosynol A, total RNAs were isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with p16, p21 and p27 primers and the reaction products were subjected to electrophoresis in a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) Cells were treated with the indicated concentrations of dideoxypetrosynol A for 48 h and collected. The cells were lysed and then cellular proteins were separated by 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the antibodies against p16, p21 and p27. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

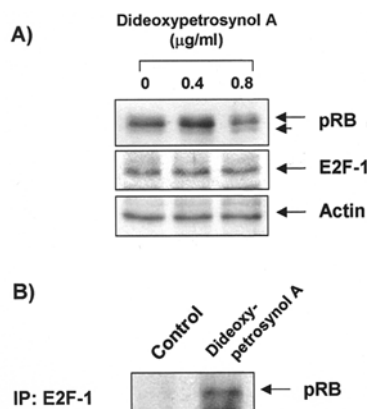


Figure 4. Hypophosphorylation of pRB and enhanced association of pRB and E2F-1 in U937 cells after exposure to dideoxypetrosynol A. (A) Cells were treated with the indicated concentrations of dideoxypetrosynol A for 48 h and total cell lysates were prepared and separated by 8% or 10% SDS-polyacrylamide gel. Western blotting was performed using anti-pRB and anti-E2F-1 antibodies. (B) Whole cell lysates (0.5 mg of protein) from control cells and cells treated with dideoxypetrosynol A were immunoprecipitated with anti-E2F-1 antibody. Immuno-complexes were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-pRB antibody. Proteins were detected by ECL detection.

A treatment without altering E2F-1 expression (Fig. 4A). Co-immunoprecipitation analysis indicated that association of pRB and E2F-1 was almost undetectable in untreated log phase cells. However, there was a strong increase in the association of pRB and E2F-1 in dideoxypetrosynol A-treated cells (Fig. 4B) suggesting that dideoxypetrosynol A inhibits the release of E2F-1 protein from pRB.

Discussion

In this study, we tested dideoxypetrosynol A, a polyacetylene from the marine sponge *Petrosia* sp., for its activity in

inhibiting the growth of human monocytic leukemia U937 cells. We found that treatment of cells with dideoxypetrosynol A resulted in a concentration-dependent inhibition of cell viability, which was associated with gross morphological changes (Fig. 1). Subsequent experiments addressed the issue of whether this compound perturbs the cell cycle using DNA flow cytometric analysis. The data revealed a cell-cycle block at G1 to S phase transition and an accumulation of cells at the sub-G1 apoptotic region (data not shown), which contained less DNA than G1 cells (18). These results suggested that dideoxypetrosynol A interferes with the proliferation of U937 cells and induces apoptosis in close association with the G1 arrest by modulation of the expression of cell cycle-regulators as a possible molecular mechanism of the effects of dideoxypetrosynol A. Thus, we investigated the effects of dideoxypetrosynol A on the expression of G1/S transition regulatory proteins to analyze the mechanism of G1 arrest.

In terms of regulation of the cell cycle, Cdks play a most critical role. Two major mechanisms for Cdk regulation are binding with its catalytic subunit cyclin followed by activation of the Cdk/cyclin complexes, and binding with Cdk inhibitors followed by inactivation of the Cdk/cyclin complexes (9,19). An alteration in the formation of these complexes could lead to increased cell growth and proliferation, or decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis (6). In general, cyclin D1 is synthesized in a pre-DNA-synthetic gap (early G1 phase) and is the key regulator of the signal transduction in G1 phase cell proliferation. If cyclin D1 is overexpressed, the checkpoint of G1/S will be out of control and lose its role in the signaling of proliferation (2,4). However, other recent results show that when cell cycle blockage occurs in the G1 phase, cyclin D1 is not decreased but accumulated in cells, suggesting that G1 arrest does not always follow the decrease of cyclin D1 expression. Also, progression through G1/S transition is regulated by cyclin E, which is expressed in late G1 preceding cyclin A expression, with maximal expression at the G1/S boundary (3,5). In the present study, the results from RT-PCR and immunoblotting analysis clearly demonstrate that the levels of cyclin D1 expression were markedly induced by dideoxypetrosynol A treatment of both transcriptional and translational levels, but the levels of cyclin E were concentration-dependently inhibited in dideoxypetrosynol A-treated U937 cells without any changes in the expression of Cdks (Fig. 2).

On the other hand, in conjunction with Cdk4/6, cyclin D mediates the initial phosphorylation of pRB. The kinase activities of cyclin E/Cdk2 and cyclin A/Cdk2 complexes then act to maintain pRB in the hyperphosphorylated state (5). Any factor affecting the activity of these kinases could abrogate the normal inactivation of pRB and cause an accumulation of cells in G1. Normally, pRB binds to the members of the E2F family of transcription factors. In response to the growth factors, pRB is phosphorylated and dissociated from E2F, which triggers G1 cell cycle progression (8,20). Thus, an obvious candidate for control of pRB phosphorylation is the cyclin E/Cdk2 complex. If decreased levels of either protein or association between respective binding partners were observed, a concomitant decrease in the degree of pRB phosphorylation would be expected (20). pRB is also known to inhibit the transcriptional activation of p16 expression in

the cells, and the genetic mutation of the RB gene resulted in high levels of p16 expression (21,22). Thus, p16 has the capacity to arrest cells in the G1-phase of the cell cycle and its probable physiological role is in the implementation of irreversible growth arrest, termed cellular senescence. Our data showed that treatment with dideoxypetrosynol A in U937 cells inhibited the phosphorylation of pRB and enhanced the association of pRB and E2F-1 (Fig. 4). Furthermore, we found that the treatment of cells with dideoxypetrosynol A selectively induced p16 expression in a dose-dependent manner, indicating that neither p21 nor p27 was involved in the G1 arrest which was induced by dideoxypetrosynol A in U937 cells.

In summary, the present study demonstrates that dideoxypetrosynol A, a polyacetylene from the marine sponge *Petrosia* sp., inhibited U937 human monocytic leukemia cell proliferation by inducing G1 cell cycle arrest. Although further studies are needed, the present study suggests that p16 and pRB play an important role in the G1 cell cycle arrest induced by dideoxypetrosynol A in human leukemia cells.

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