

Inhibitory Effect of Human Breast Cancer Cell Proliferation *via* p21-Mediated G₁ Cell Cycle Arrest by Araliadiol Isolated from *Aralia cordata* Thunb.

Authors

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Key words

- araliadiol
- cytotoxicity
- cell cycle
- G₁ arrest
- *Aralia cordata*
- Araliaceae

Abstract

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A new polyacetylenic compound, araliadiol, was isolated from the leaves of *Aralia cordata* Thunb. (Araliaceae). The structure of araliadiol was determined to be 3(S),8(R)-pentadeca-1,9(Z)-diene-4,6-diyne-3,8-diol by MS, NMR, IR, and UV spectroscopic analysis as well as Mosher ester reaction. Araliadiol displayed a significant inhibitory effect on the growth of a human breast adenocarcinoma cell line (MCF-7), with an IC₅₀ value for cytotoxicity of 6.41 μg/mL. Cell cycle analysis revealed that the proportion of cells in the G₁ phase of the cell cycle increased in a dose-dependent manner (from 54.7% to 72.0%) after 48 h exposure to araliadiol at dosages ranging from 0 to 80 μM. The results suggest that araliadiol inhibits cell cycle progression of MCF-7 at the G₁-S transition. After treatment with araliadiol, phosphorylation of retinoblastoma protein (Rb) in MCF-7 cells was inhibited, accompanied by a decrease in the levels of cyclin D₃ and cyclin-dependent kinase 4 (cdk4) and an increase in the expression of p21^{WAF-1/Cip1}.

However, the expression of phosphorylated p53 (Ser15) and Chk2 was not altered in MCF-7 cells. These findings indicate that araliadiol exhibits its growth-inhibitory effects on MCF-7 cells through downregulation of cdk4 and cyclin D₃, and upregulation of p21^{WAF-1/Cip1} by a p53-independent mechanism.

Abbreviations

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MCF-7:	human breast adenocarcinoma cell line
IC ₅₀ :	half maximal inhibitory concentration
cdk:	cyclin-dependent kinase
CKIs:	cdk inhibitors
MTT:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Rb:	retinoblastoma protein
MTPA:	α-methoxy-α-trifluoromethyl-phenylacetyl
DMAP:	dimethylaminopyridine
PI:	propidiumiodide

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Introduction

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Aralia cordata Thunb. (Araliaceae) is a medicinal herb distributed at medium to high altitudes in the central range of Taiwan [1]. A number of polyacetylenes have been isolated from this family [2] and have been shown to possess cytotoxic [3,4] and anti-inflammatory activities [5,6]. In addition, some polyacetylenes such as panaxydol [7] and panaxytriol [8] have been demonstrated to exhibit anticancer activity by inducing G₁ and G₂/M cell cycle arrest, respectively.

The cell cycle is regulated by members of the cyclin family of proteins, which bind and activate members of the cyclin-dependent kinase (cdk) family to effect cell cycle progression. Progression of cells through the G₁-S-G₂-M cycle is followed

by successive oscillations in the levels of cyclins D, E, A, and B [9]. Cyclin D and cyclin E-cdk complexes phosphorylate the Rb protein which plays an essential role in regulation of the passage through the G₁ phase into S. Regulation of cyclin-cdk complexes activity is controlled at three levels. First, expressions of cdks and cyclins are altered. Second, the cdk kinase activity is regulated by reversible phosphorylation. Finally, cdk inhibitors (CKIs), such as p21^{WAF-1/Cip1} and p27^{KIP}, interact reversibly and prevent kinase activation [10].

In this study, we isolated a new polyacetylene, araliadiol, from *A. cordata*. We show that araliadiol has a potent cytotoxicity against human breast carcinoma cells (MCF-7 cells) and demonstrate that treatment of MCF-7 cells with aralia-

diol results in growth arrest that is mediated by p21^{WAF-1/Cip1} and independent of p53. Hence, araliadiol has chemotherapeutic potential for suppressing tumor growth in p53-deficient malignancies.

Materials and Methods

General experimental procedures

Optical rotation, UV, and IR data were acquired on a PerkinElmer 241 polarimeter, a Bio-Tek μ Quant MQX200 ELISA reader, and a PerkinElmer Spectrum 100 FT-IR spectrometer, respectively. ¹H-NMR, ¹³C-NMR, HSQC, HMBC, ¹H-¹H gCOSY, and DEPT spectra were obtained on a Varian Unity Inova-600 MHz spectrometer using CDCl₃ as the solvent. HREIMS data were determined with a Thermo/Finnigan Quest MAT 95XL Mass Spectrometer. HPLC was carried out over a Phenomenex column (5 μ m, 250 \times 10 mm, Phenomenex Co.) using a Shodex RI-101 detector. Open column chromatography was carried out over silica gel (4 \times 30 cm; 60–80 mesh; Merck).

Plant material

The leaves of *A. cordata* were collected in July 2009 from Tsuifeng, Nantou County, Taiwan, and were identified by Dr. Yen-Hsueh Tseng (NCHU). The voucher specimen [TCF13524] was deposited in the herbarium of the same university.

Extraction and purification

Air-dried leaves of *A. cordata* (242 g) were extracted with EtOH (10 L) at ambient temperature and concentrated under vacuum to yield the EtOH extract (23.88 g). The EtOH extract was partitioned between EtOAc-H₂O to give the EtOAc-soluble fraction (13.25 g) and the H₂O-soluble fraction. The EtOAc-soluble fraction displayed potent cytotoxicity (IC₅₀ = 27.3 μ g/mL) and was further chromatographed over silica gel (4 \times 30 cm; 60–80 mesh; Merck) eluted with *n*-hexane and a gradient of *n*-hexane-EtOAc (100:0; 98:2; 95:5; 92:8; 89:11; 86:14; 83:17; 80:20; 75:25; 70:30; 65:35; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100, each 2 L). The eluent was collected in constant volumes (each 500 mL), and combined into 19 fractions based on TLC properties. Fraction 7 (obtained with *n*-hexane:EtOAc = 89:11, amount = 5.3 g) displayed the strongest cytotoxicity (IC₅₀ = 10.9 μ g/mL) and was further separated by HPLC using a normal-phase column (250 \times 10 mm, 5 μ m, Phenomenex Co.) with a mixture of *n*-hexane:EtOAc = 75:25 as eluent at a flow rate of 2 mL/min to obtain compound araliadiol (0.25 g).

Araliadiol (1): yellow oil; [α]_D²⁵ = 198.8° (*c* 0.89, CHCl₃); UV (MeOH) λ_{max} (log ϵ) = 231, 244, 257 nm; IR ν_{max} = 3306, 3012, 2954, 2924, 2855, 2231, 2150, 1644, 1456, 1378, 1301, 1265, 1117, 984, 931, 879, 783 cm⁻¹; ¹H NMR (600 MHz, CDCl₃): δ = 5.47 (1H, *d*, *J* = 16.8 Hz), 5.25 (1H, *d*, *J* = 10.2 Hz), 5.94 (1H, *ddd*, *J* = 5.4, 10.2, 16.8 Hz), 4.94 (1H, *d*, *J* = 5.4 Hz), 5.20 (1H, *d*, *J* = 8.4 Hz), 5.51 (1H, *dd*, *J* = 10.2, 8.4 Hz), 5.60 (1H, *ddd*, *J* = 7.8, 10.2 Hz), 2.10 (2H, *q*, *J* = 7.8 Hz), 1.38 (2H, *m*), 1.23–1.31 (4H, *m*), 0.89 (3H, *t*, *J* = 6.6 Hz); ¹³C NMR (150 MHz, CDCl₃): δ = 117.3 (C-1), 135.7 (C-2), 63.3 (C-3), 78.2 (C-4), 70.2 (C-5), 68.8 (C-6), 79.8 (C-7), 58.4 (C-8), 127.5 (C-9), 134.5 (C-10), 27.6 (C-11), 29.1 (C-12), 22.6 (C-13), 31.7 (C-14), 14.1 (C-15); EIMS *m/z* (rel. int) = 232.2 (M⁺), HREIMS: C₁₅H₂₀O₂ found 232.1470[M⁺], calc.: 232.1463.

Preparation of Mosher's esters

Absolute configuration of compound **1** at C-3 and C-8 was determined by the modified Mosher's method [11]. Briefly, compound **1** was dried under vacuum for 1 h, and then two portions (4 mg each) were dissolved in dry CH₂Cl₂ (2 mL) containing 2 mg of dimethylaminopyridine (Alfa Aesar). Eight equivalents of dicyclohexylcarbodiimide (Alfa Aesar) were added to each portion of araliadiol. Six equivalents of either the (*S*) or the (*R*) isomer of *a*-methoxy-*a*-trifluoromethylphenylacetyl chloride (Alfa Aesar) were added to a portion of araliadiol, resulting in the formation of the (*R*) and (*S*) Mosher's esters of araliadiol, respectively. The reaction mixtures were stirred for 24 h at room temperature, concentrated on a rotary evaporator and then redissolved in diethyl ether (3 mL). The reaction mixtures were partitioned between H₂O and ether, and extracts were concentrated and purified by HPLC. ¹H NMR spectrum was recorded, and differences in the signals of protons that neighbored the chiral centers noted. The bulk effect on chemical shifts induced by esterification with the chiral reagents was calculated using $\Delta\delta$ (= δ_S - δ_R), where δ_S and δ_R are the shifts (in ppm) of diagnostic protons neighboring the chiral centers in araliadiol of the (*S*) and (*R*) Mosher's esters, respectively. The absolute configuration of araliadiol was obtained by positioning the protons with positive $\Delta\delta$ values on the right side and those with negative $\Delta\delta$ values on the left side in the model (● Fig. 1 B).

Cell culture and proliferation assay

MCF-7 (human breast adenocarcinoma, BCRC 60436) was purchased from BCRC (Bioresource Collection and Research Center), Food Industry Research, and Development Institute, Taiwan. MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 mM sodium pyruvate, and were maintained at 37°C and 5% CO₂. All cells (1 \times 10³ per well) were seeded in 96-well plates and incubated for 24 h, and different dosages of extracts of araliadiol were added to each well in triplicate for 5 days. The cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [12]. Plumbagin (Sigma) was used as positive control.

Cell cycle analysis

MCF-7 cells, seeded in a 60-mm dish (2 \times 10⁵ cells/dish), were treated with 20 μ M, 40 μ M, and 80 μ M araliadiol for 48 h or with 40 μ M araliadiol for 0, 6, 12, 18, 24, and 48 h. Subsequently, cells were trypsinized, collected with ice-cold PBS and resuspended in 1 mL PBS. Cells were then fixed by the addition of 3 mL ice-cold 95% ethanol at -20°C overnight. The cell pellets were collected by centrifugation and rinsed with ice-cold PBS. Cells were stained with 1 mL of 50 μ g/mL propidium iodide (PI) in hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 mg/mL RNase A) for 30 min. Fluorescence emitted from the PI-DNA complex was quantified after excitation of the fluorescent dye by flow cytometry (Cytomics FC 500, Beckman Coulter).

Protein expression analysis

The protein expression after treatment with araliadiol was determined by a Western blotting assay. Briefly, MCF-7 cells incubated in 100-mm culture dishes (1 \times 10⁶ cells per dish) and were treated with araliadiol at the dosages of 20 μ M, 40 μ M, and 80 μ M for 48 h. 40 μ g of total cell proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane. Detection was performed by immunostaining using specific primary antibodies and horse-

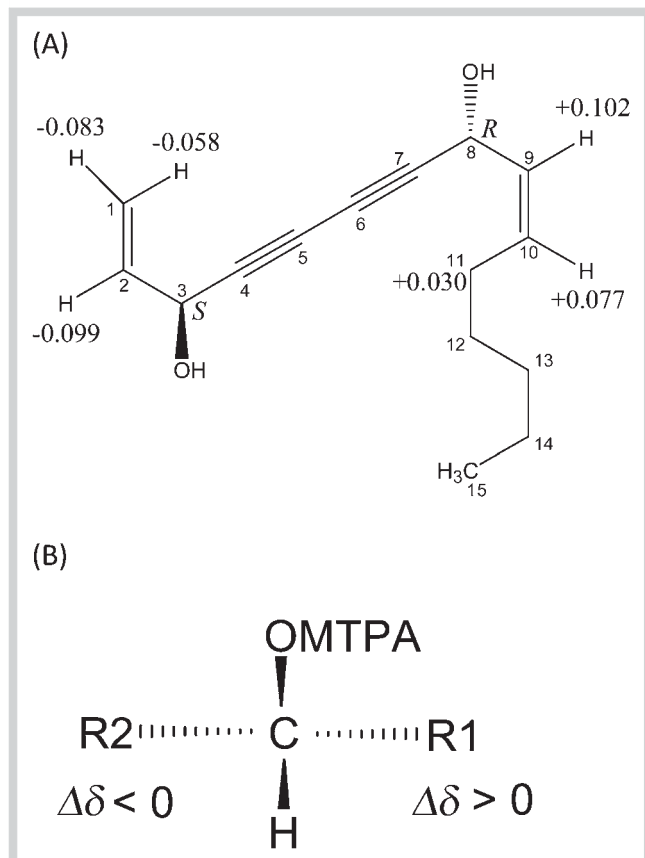


Fig. 1 A and B A Structure of araliadiol depicting its 3-(*S*),8-(*R*) absolute stereochemistry. The numbers represent $\Delta\delta$, where $\Delta\delta = \delta_S - \delta_R$. δ_S and δ_R are the chemical shift values for a particular group of the (*S*) and (*R*) Mosher's esters of araliadiol. B Model used to determine the absolute configurations of secondary alcohols.

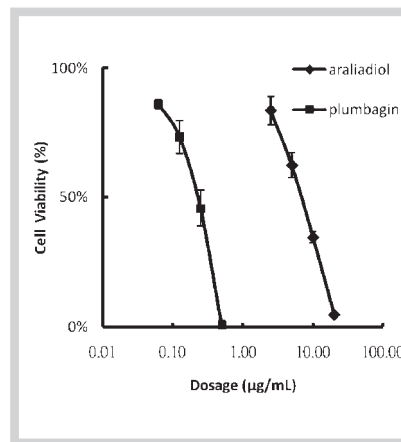


Fig. 2 Cytotoxic activity of araliadiol and plumbagin in MCF-7 cells. Cells were treated with various concentrations (2.5–20 $\mu\text{g/mL}$) of araliadiol for 5 days. The data are presented in terms of proportional viability (%) by comparing the araliadiol- and plumbagin-treated groups with untreated cells, the viability of which was assumed to be 100%.

radish peroxidase-conjugated anti-IgG antibody. The proteins were detected by chemiluminescence (ECL, Pierce Biotechnology, Inc.). The following antibodies were used for the Western blots: rabbit polyclonal antibodies to p27^{Kip1}, cdk2, phospho(Ser807/811)-Rb, and Chk2; mouse polyclonal antibodies to cdk6, cdk4, cyclin D1, cyclin D3, cyclin E, p21^{Waf1/Cip1}, phospho(Ser15)-p53, and β -actin; goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase-conjugate, and horse anti-mouse immunoglobulin G (IgG)-horseradish peroxidase-conjugate. Antibodies were used at working dilutions of 1 : 1000 with the exception of antibodies to β -actin, for which a working dilution of 1 : 10000 was employed.

Statistical analysis

Data are expressed as means \pm SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (* $p < 0.05$) between the control (untreated) and treated cells were analyzed by Dunnett's test.

Results and Discussion

To investigate the cytotoxicity of *A. cordata*, we examined the effect of ethanolic extracts against cell proliferation in MCF-7 cells by using the standard MTT method [12]. Data showing the inhibitory effects of the extract and derived fractions on the

growth of MCF-7 cells are presented in the "Extraction and Purification" section. A yellow oil compound (**1**) was isolated from the strongest cytotoxicity subfraction (fraction 7). The HREIMS spectrum for compound **1** gave an ion peak [M^+] at 232.1470, suggesting a molecular formula of $C_{15}H_{20}O_2$. The UV absorptions at 231, 245, and 257 nm were similar to published data for diacetylenes [13], whereas the IR spectrum had absorptions corresponding to hydroxyl groups (3360 cm^{-1}), double bonds (1644 cm^{-1}), and triple bonds (2231 and 2150 cm^{-1}), respectively. In the ^1H NMR spectrum, terminal olefin protons at δ_{H} 5.94 (H-2), 5.47, and 5.25 (H-1), another olefinic signal at δ_{H} 5.60 (H-10), 5.51 (H-9), two oxymethines at δ_{H} 5.20 (H-8) and at δ_{H} 4.94 (H-3), besides methylene protons at δ_{H} 2.10 (H-11), 1.38 (H-12), and 1.23–1.31 (H-13, H-14) and methyl protons at δ_{H} 0.89 (H-15) were observed (Table 1). The configuration of the two olefin protons (H-9 and H-10) was assigned as a *Z* configuration based on their coupling constants ($J_{9,10} = 10.2\text{ Hz}$). Furthermore, analyses of the ^1H , ^{13}C NMR, and ^1H - ^1H COSY spectra permitted establishment of the sequence C-8–C-15 of the molecule, which was characterized by an alcohol function (C-8), a *cis* double bond (C-9 and C-10), four methylenes (C-11–C-14), and a methyl group (C-15), respectively. Further NMR spectroscopic analysis resulted in the determination of the remaining part of the molecule, namely a terminal olefin (C-1 and C-2) and a hydroxy group (C-3). In the ^{13}C NMR spectrum, four quaternary carbon signals at δ_{C} 79.8 (C-7), 78.2 (C-4), 70.2 (C-5), and 68.8 (C-6) were characteristically observed (Table 1). Furthermore, long-range correlations of H-2 to C-4, H-3 to C-4 and C-5, H-8 to C-6 and C-7, and H-9 to C-7 suggested that C-1–C-3 and C-8–C-15 were linked by a conjugated diyne moiety (C-4–C-7). Therefore, the structure of compound **1** was elucidated as heptadeca-1,9(*Z*)-diene-4,6-diyne-3,8-diol, and named araliadiol. In order to determine the absolute configurations at C-3 and C-8, a modified Mosher's method [11] was applied. Araliadiol was treated with (*R*)- and (*S*)- α -methoxy- α -trifluoromethylphenylacetyl chloride (MTPA-Cl) in the presence of 4-dimethylaminopyridine (DMAP) and dicyclohexylcarbodiimide to give the di-(*S*)- and di-(*R*)-MTPA esters respectively. In the ^1H NMR spectrum of the di-(*S*)-MTPA ester, the proton signals assigned to H-1, and H-2 were observed at a higher field than those of the di-(*R*)-MTPA ester, and H-9, H-10, and H-11 were observed at a lower field than those of the di-(*R*)-MTPA ester, indicating that C-3 and C-8 had *S*- and *R*-configurations respectively (Fig. 1).

As shown in Fig. 2, when MCF-7 cells were treated with araliadiol at dosages of 2.5–20 $\mu\text{g/mL}$, a dose-dependent decrease of cell viability was observed. The IC_{50} value of araliadiol against

Table 1 ^1H -NMR and ^{13}C -NMR spectral data for araliadiol (**1**) and its *S*- and *R*-MTPA mosher esters.

Carbon number	^1H (J_{HH} in Hz)	^{13}C	$\delta\text{S-ester}$	$\delta\text{R-ester}$	$\Delta\delta$ (= $\delta\text{S} - \delta\text{R}$)
1	5.47 (1H, <i>d</i> , 16.8) 5.25 (1H, <i>d</i> , 10.3)	117.3	5.512	5.595	-0.083
2	5.94 (1H, <i>ddd</i> , 5.4, 10.2, 16.8)	135.7	5.812	5.911	-0.099
3	4.94 (1H, <i>d</i> , 5.4)	63.3			
4		78.2			
5		70.2			
6		68.8			
7		79.8			
8	5.20 (1H, <i>d</i> , 8.4)	58.4			
9	5.51 (1H, <i>dd</i> , 10.2, 8.4)	127.5	5.543	5.441	+0.102
10	5.60 (1H, <i>ddd</i> , 7.8, 10.2)	134.5	5.762	5.685	+0.077
11	2.10 (2H, <i>q</i> , 7.8)	27.6			
12	1.38 (2H, <i>m</i>)	29.1			
13	1.23–1.31 (4H, <i>m</i>)	22.6			
14		31.7			
15	0.89 (3H, <i>t</i> , 6.6)	14.1			

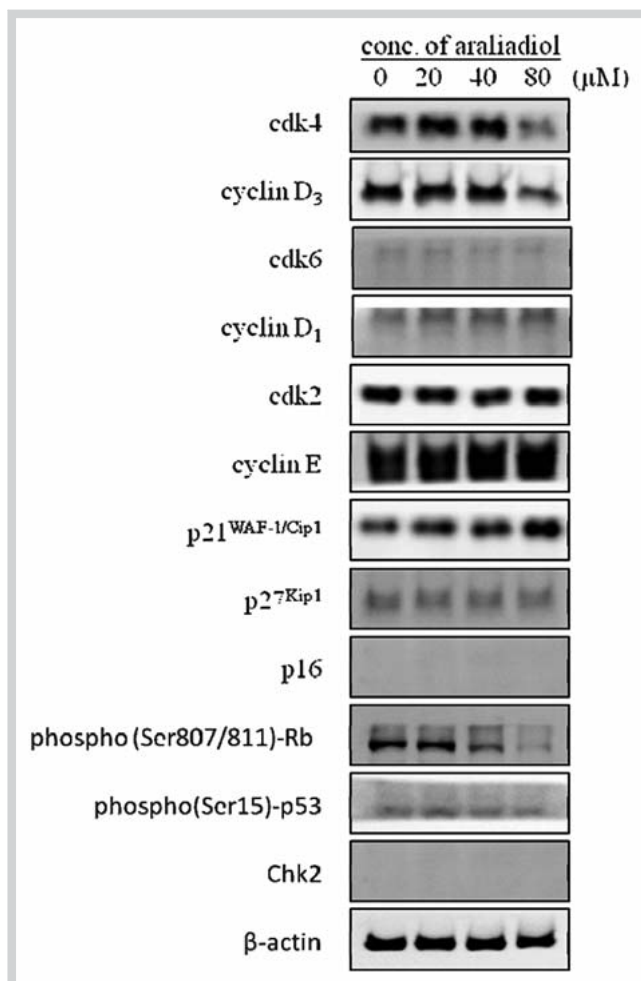
Table 2 Cell cycle distribution of MCF-7 cells treated with araliadiol.

Treatment	Percentage of cells in			
	G ₁	S	G ₂ /M	SubG ₁
Control	54.6 ± 0.7	18.2 ± 0.3	21.7 ± 1.1	1.1 ± 0.2
Araliadiol				
▶ 20 μM	68.2 ± 0.2*	12.7 ± 0.2*	14.6 ± 0.6*	1.1 ± 0.1
▶ 40 μM	75.2 ± 1.0*	9.3 ± 0.8*	12.1 ± 0.5*	1.3 ± 0.3
▶ 80 μM	72.0 ± 2.4*	11.4 ± 0.2*	13.2 ± 1.9*	1.3 ± 0.1
Plumbagin (positive control)				
4 μM	56.7 ± 2.0	12.3 ± 0.7*	11.2 ± 1.2*	17.3 ± 3.7*

* $P < 0.05$ versus control by analysis of variance with Dunnett's post hoc test

MCF-7 cells was 6.41 μg/mL (27.6 μM). Although cytotoxicity of araliadiol was weaker than that of plumbagin ($\text{IC}_{50} = 0.24$ μg/mL) used as a positive control, comparison with the other polyacetylenic compounds, panaxytriol and dehydrofalcariindiol-8 acetate (IC_{50} values > 100 μM) [8, 14], revealed that the growth-inhibiting activity of araliadiol was potent. To examine the mechanism responsible for araliadiol-mediated cell growth inhibition, cell cycle distribution was evaluated using flow cytometric analysis. When MCF-7 cells were treated with 0, 20, 40, and 80 μM of araliadiol for 48 h, the percentage of G₁ cells was 54.6, 68.2, 75.2, and 72.0, respectively (▶ **Table 2**). The results showed that treating cells with araliadiol caused a significant inhibition of cell cycle progression in MCF-7 cell lines at 48 h, resulting in a clear increase in the percentage of cells in the G₁ phase when compared with the control, which is consistent with G₁ arrest.

Next, we performed Western blot analysis to examine the effects of araliadiol on the expression of G₁-specific cell cycle regulation proteins. When MCF-7 cells were exposed to different doses of araliadiol for 48 h, the levels of cyclin D₃ and cdk4, two major G₁ regulatory proteins, decreased significantly at high concentration (80 μM) treatment (▶ **Fig. 3**). By contrast, protein levels of cdk6, cdk2, cyclin D₁ and cyclin E were found to be unaltered by araliadiol treatment. In addition, exposure of cells to araliadiol resulted in a dose-dependent increase in the levels of tumor suppressor

**Fig. 3** The expression of cell cycle-related proteins in MCF-7 cells treated with various concentrations of araliadiol was determined by Western blotting.

[12] p21^{Waf1/Cip1}, a cyclin-dependent kinase inhibitor belonging to the Cip/Kip family (p21^{Waf1/Cip1}, p27^{Kip1}, and p57^{Kip2}), and there is no change in p27^{Kip1} expression. Meanwhile, the p16, a cdk4/ cdk6 inhibitor, was detected in MCF-7 cells, which is in agreement with the deletion of the MTS1 gene that encodes p16 [15, 16] in this cell line. The level of Rb (phosphorylation at Ser807/811), a major regulator of the G₁/S transition, was reduced in MCF-7 cells by araliadiol treatment. According to previous studies, Rb binds to and represses the transcription factor E2F during early and mid-G₁ phase [17], and can be phosphorylated by G₁ cyclin-cdk complexes in the late G₁ phase [18, 19]. Phosphorylated Rb releases E2F, permitting the transcription of the S-phase promoting gene [20, 21]. In araliadiol-treated cells, inhibition of cdk activity by a combination of cdk4 and cyclin D₃ downregulation and p21^{Waf1/Cip1} upregulation would therefore be expected to prevent changes in E2F-regulated gene expression following the failure of these cells to progress from G₁ into S.

Expression of p21^{WAF-1/Cip1} can be regulated by p53-dependent or p53-independent mechanisms [22–25]. Previous studies indicated that G₁/S arrest could be achieved by p53, which is phosphorylated on Ser15 by ATM/ATR and on Ser20 by Chk2, through induction of p21^{WAF-1/Cip1} transcription [26]. Because no altered expression of phosphorylated p53 (Ser15) and no expression of Chk2 were detected in MCF-7 cells under araliadiol treatment,

our data indicate that p21 is upregulated by a p53-independent mechanism.

Based on the results obtained in this study, we conclude that araliadiol, a new polyacetylene isolated from *A. cordata*, possesses a potent cytotoxicity activity against MCF-7 cells.

The antiproliferation effect of araliadiol on MCF-7 cells occurs through downregulation of cdk4 and cyclin D₃ and upregulation of p21^{WAF-1/Cip1} by a p53-independent mechanism. Further study is needed to evaluate its antitumor activity.

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