Imbricatolic Acid from *Juniperus communis* L. Prevents Cell Cycle Progression in CaLu-6 Cells

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Key words
- *Juniperus communis*
- Cupressaceae
- diterpenes
- dihydrobenzofuran lignan glycoside
- CDK inhibitors
- cell cycle

Abstract

Imbricatolic acid was isolated from the methanolic extract of the fresh ripe berries of *Juniperus communis* (Cupressaceae) together with sixteen known compounds and a new dihydrobenzofuran lignan glycoside named juniperoside A. Their structures were determined by spectroscopic methods and by comparison with the spectral data reported in literature.

Imbricatolic acid was evaluated for its ability to prevent cell cycle progression in p53-null CaLu-6 cells. This compound induces the upregulation of cyclin-dependent kinase inhibitors and their accumulation in the G1 phase of the cell cycle, as well as the degradation of cyclins A, D1, and E1. Furthermore, no significant imbricatolic acid-induced apoptosis was observed. Therefore, this plant-derived compound may play a role in the control of cell cycle.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Several medicinal plants, such as *Juniperus* sp. (Cupressaceae) [1], contain metabolites able to induce cell cycle arrest, playing an important role in cancer prevention. Many reports are focused on the determination of the chemical composition of the berry's essential oil [2,3], and limited data are reported on nonvolatile components. Chemical components of the methanol extract from *Juniperus communis* berries include labdane monoterpene and megastigmane glycosides [4], labdane diterpenes [5], flavonoids, and biflavonoids [6]. Labdane diterpenes, isolated from several plant families, show a variety of biological activities [7] including the inhibition of cell proliferation in several cell lines [8]. In this study we analyzed the methanolic extract of berries from *J. communis* and focused on the bioactivity of imbricatolic acid, a labdane diterpene isolated as a major component.

Cyclin/cdk (cyclin dependent kinase) complexes facilitate progression through the cell cycle and are activated at specific checkpoints [9]. These complexes are also regulated by their binding to CDK inhibitors (CKIs) [10]. Two CKI gene families have been defined. The INK4 gene family encodes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, all of which bind to CDK4 and CDK6 and inhibit kinase activities by interfering with their association with D-type cyclins [9]. In contrast, CKIs of the Cip/Kip family ($p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$, and $p57^{Kip2}$) bind to both cyclins and CDK subunits and can modulate the activities of cyclin D-, E-, A-, and B-CDK complexes [9]. $p21^{Cip1/Waf1/Sdi1}$ gene expression is regulated by transcriptional and posttranscriptional mechanisms. In tumor cells which lack p53 or with a mutant form of p53, $p21^{Cip1/Waf1/Sdi1}$ is activated through p53-independent pathways.

In the present paper, we report: i) the isolation and structural elucidation of a new compound and eleven known components from a methanolic extract of berries from *J. communis*, and ii) the ability of imbricatolic acid, a known labdane diterpene, to induce CKIs upregulation, accumulation in G1 phase of the cell cycle and cyclins degradation in p53-null human lung tumor cells.

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Materials and Methods

General experimental procedures

High-resolution fast atom bombardment mass spectrometry (HRFAB-MS) was recorded on a Fisons VG Prospec instrument, and electrospray ionization mass spectrometry (ESI-MS) experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. Optical rotations were determined on a Jasko P-2000 polarimeter. ^1H and ^{13}C NMR spectra were determined on a Varian Unity INOVA spectrometer at 500.13 and 125.77 MHz, respectively, equipped with an indirect detection probe. Chemical shifts were referenced to the solvent signals: deuterated methanol (CD_3OD). GC analyses were performed on an Agilent Technologies 6850 Series II gas chromatograph for capillary column (HP-5, 30 m × 0.25 mm, 180 °; helium carrier flow 10 mL min^{-1}) and a FID detector operated at 260 °. Droplet countercurrent chromatography (DCCC) was performed on a DCC-A apparatus (Tokyo Rikakikai Co.). HPLC was performed using a Waters 510 pump equipped with a Waters U6K injector and a Waters 401 differential refractometer as the detector, using a C_{18} μ-Bondapak column (30 cm × 3.9 mm; i.d.; flow rate 1 mL min^{-1}; Waters) and a Luna C-18 column (3 μ, 150 × 4.60 mm i.d.; flow rate 1 mL min^{-1}; Phenomenex).

Plant material

Ripe berries of *Juniperus communis* L. (Cupressaceae) were collected in the mountain areas of Isernia (Italy) in October 2008 and identified by Dr. Paola Fortini. A voucher specimen is deposited (JC-486-08) at the Herbarium of DiSTAT, University of Molise (Pesche). Berries were kept frozen at −20 °C until analyzed.

Extraction and isolation

Fresh ripe berries (400 g) were crushed and extracted with MeOH (3 × 2 L) at room temperature for 24 h. The combined extracts (170 g) were concentrated and subjected to a modified Kupchan's partition methodology as described [11]. The MeOH extract was dissolved in 10% aqueous methanol and partitioned against *n*-hexane (3 × 400 mL) yielding 1.3 g of extract. The water content (% v/v) of the MeOH extract was adjusted to 40% and partitioned against $CHCl_3$ (5 × 400 mL), yielding 1.5 g of extract; the aqueous residue was concentrated and partitioned against *n*-BuOH (3 × 500 mL) to give 2.0 g of *n*-BuOH extract. The *n*-hexane extract (1.3 g) was separated by column chromatography on SiO_2 (50 g, 230–400 mesh silica gel; 1.5 × 45 cm) and stepwise eluted using *n*-hexane/EtOAc with the ratio of 100 : 0 (300 mL), 99 : 1, 98 : 2, 80 : 20 (each 150 mL), 96 : 4, 95 : 5, 50 : 50, 0 : 100 (each 200 mL) to give 8 corresponding fractions (A–H). 150 × 10 mL tubes were collected and combined on the basis of their similar TLC behavior (SiO_2 with *n*-hexane/EtOAc 95 : 5). Fraction H (70 mg) was then purified by HPLC (C_{18} μ-Bondapak column; MeOH/H_2O 8 : 2 as eluent, flow rate 1 mL/min) to give mainly *cis*-communic acid (**6**, 2.8 mg). The $CHCl_3$ extract (1.5 g) was fractionated by DCCC using $CHCl_3$/MeOH/H_2O (7 : 13 : 8) in the ascending mode (the lower phase was the stationary phase); 320 tubes (6 mL) were collected, flow rate 18 mL/h. Fractions were combined and monitored by TLC on SiO_2 with $CHCl_3$/MeOH/H_2O (80 : 18 : 2) as eluent to give five main fractions summarized in **Table 1**. The *n*-BuOH extract (2 g) was submitted to DCCC with *n*-BuOH/Me_2CO/H_2O (3 : 1 : 5) in the descending mode (the upper phase was the stationary phase). 210 tubes (6 mL) were collected, flow rate 18 mL/h. Fractions were combined and monitored by TLC on Silica gel plates with *n*-BuOH/OHAc/H_2O (12 : 3 : 5) and $CHCl_3$/MeOH/H_2O (80 : 18 : 2). Five fractions (A–E) were obtained and purified by HPLC as summarized in **Table 1**. The purity of compounds **1**–**17** was greater than 95% determined by HPLC method (see experimental section), MS, and NMR. The bold number in brackets refers to the chemical structures indicated in **Fig. 1**. Copies of original spectra can be obtained from the author of correspondence.

Table 1 DCCC fractionation and HPLC purification of $CHCl_3$ and *n*-BuOH extracts.

Fraction	Amount (mg)	Compound	MeOH/H_2O
	$CHCl_3$ extract[a]		
1	1.2	**17**	1 : 1
2	1.5	**16**	7 : 3
2	1.7	**8**	8 : 2[b]
3	5.8	**2**	7 : 3
3	1.8	**4**	45 : 55[b]
4	3.0	**10**	8 : 2
4	1.0	**11**	8 : 2
4	1.2	**12**	8 : 2
5	1.8	**9**	75 : 25
5	1.0	**5**	75 : 25
5	2.0	**3**	75 : 25
	***n*-BuOH extract**[a]		
A	1.3	**7**	
B	1.4	**1**	4 : 6
C	3.2	**15**	1 : 1
D	1.3	**14**	8 : 2
E	3.0	**13**	1 : 1

[a] All fractions were purified on C18 μ-Bondapak column. [b] Fraction purified on a Luna C-18 column

Acid hydrolysis of 1

Compound **1** (0.5 mg) was hydrolyzed with 2N CF_3CO_2H (2 mL) at 110 ° in a sealed tube for 8 h. After cooling, the solution was diluted with H_2O (5 mL) and extracted with AcOEt (3 × 2 mL). The aqueous layer was evaporated to dryness under reduced pressure, and the residue was reacted with 0.1 M L-cysteine methyl ester hydrochloride in anhydrous pyridine (200 μL) for 1 h at 60 ° [12]. 1-(Trimethylsilyl)imidazole in pyridine was added, and the thiazolidine derivatives analyzed by GC. L-rhamnose was confirmed in **1** by comparison of the retention time of their derivatives with those of D-rhamnose (t_R = 12.60 min) and L-rhamnose (t_R = 12.14 min).

Reagents and cell culture treatments

SDS-PAGE reagents were from Bio-Rad. Anti-active phosphorylated ERK1/2, anti-tubulin, anti-PKCδ, anti-cyclinA, anti-cyclinB, anti-cyclinD1, anti-cyclinE1, anti- $p21^{Cip1/Waf1/Sdi1}$, anti-$p27^{Kip}$, anti-$p16^{INK4a}$, anti-EGFR, anti-PARP-1, anti-caspase3, and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology Inc. Protein A-horseradish peroxidase and anti-mouse Ig-horseradish peroxidase were from Amersham Pharmacia. PD098059 and rottlerin were from Calbiochem. PepTag® assay for nonradioactive detection of protein kinase C was from Promega. Human anaplastic lung cancer cells CaLu-6 were purchased from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% L-glutammin, and 1% modified Eagle's medium (MEM).

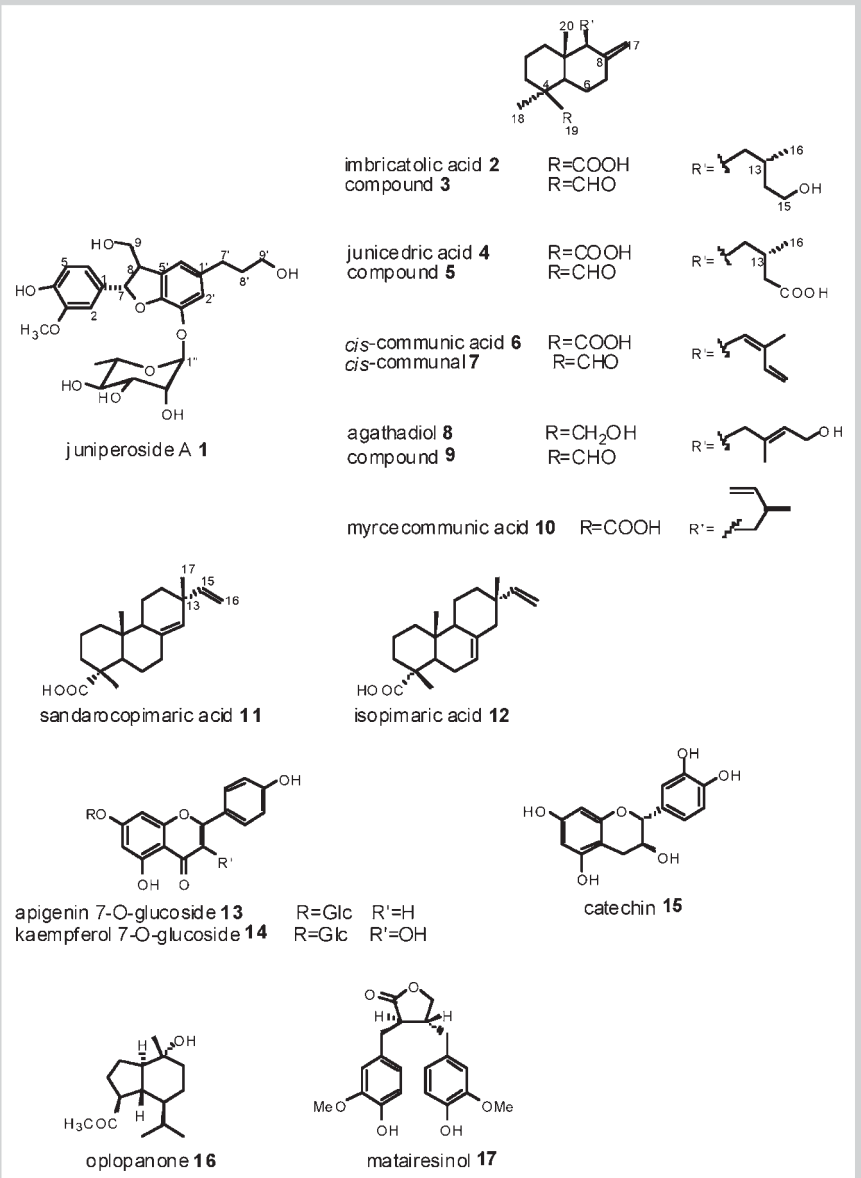

Fig. 1 Natural compounds isolated from *Juniperus communis* L. berries.

Imbricatolic acid was diluted in DMSO at a final concentration of 10 mM. Cells were grown until they reached 80% confluence and successively stimulated by imbricatolic acid at the final concentration of 10 µM for different times, as indicated in the figures. In other experiments, growing cells were preincubated with 50 µM PD098059 for 90 min, or with 1 µM rottlerin for 1 h, before the stimulation with 10 µM imbricatolic acid.

Western blot analysis and protein kinase C activity assay

Total cellular lysates and cell membranes were purified from Ca-Lu-6 cells as previously described [13]. Western blot experiments were performed in triplicate, as previously described [13]. The expression of targeted proteins was detected by an ECL kit (Amersham Biosciences) and visualized by autoradiography. Protein kinase C activity assay was performed accordingly to manufacturer's instructions (Materials and Methods **1S**, Supporting Information).

Cell viability

Calu-6 cells were placed (4×10^4 cells per well) in 96-well plates (Corning) and incubated at 37 °C in 200 µL of complete culture medium containing 10 µM imbricatolic acid or 0.1% DMSO for 3, 12, 24, 48, and 72 hrs. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT; 5 mg/mL in PBS) was added to each well and incubated for 4 h. After removal of the medium, 0.2 mL of DMSO was added to each well. The absorbance of the resulting formazan salts was recorded on a microplate reader at the wavelength of 540 nm. The effect of imbricatolic acid on growth inhibition was assessed as percent cell viability where DMSO-treated cells were taken as 100% viable. DMSO at the concentrations used was without any effect on cell viability. Four independent experiments were performed.

Analysis of apoptosis and cell cycle

Analysis of DNA content by propidium iodide incorporation was performed in permeabilized cells, as described [14] (Materials and Methods **2S**, Supporting Information).

Table 2 ^{1}H and ^{13}C NMR data (CD_3OD, 500 and 125 MHz) of compound **1**.

Position	δ_H (*J* in Hz)	δ_C	HMBC
1	–	138.8	
2	7.06 br s	110.9	C4, C6, C7
3	–	151.7	
4	–	146.1	
5	7.08 d (8.4)	119.2	C1, C3
6	6.94 d (8.4)	118.7	C2, C4, C7
7	5.57 d (5.7)	89.0	C1, C2, C6, C8, C9, C3′, C5′
8	3.47 ovl	55.6	
9	3.77 dd (9.9, 17.9), 3.88 ovl	64.8	C7, C8, C5′
1′	–	136.4	
2′	6.59 s	116.7	C4′, C6′, C7′
3′	–	146.0	
4′	–	140.7	
5′	–	129.1	
6′	6.62 s	116.3	C8, C2′
7′	2.58 d (7.3)	32.4	C1′, C2′, C6′, C8′, C9′
8′	1.80 m	35.5	C1′, C7′, C9′
9′	3.58 t (6.5)	62.0	C7′, C8′
OCH_3	3.83 s	56.0	C5
Rhamnose			
1′′	5.35 br s	101.1	C3′, C3′′, C5′′
2′′	4.08 br s	71.7	
3′′	3.90 ovl	71.9	
4′′	3.47 ovl	73.5	
5′′	3.82 ovl	70.5	
6′′	1.22 d (6.2)	17.6	C4′′, C5′′

^{1}H and ^{13}C assignments achieved by COSY, TOCSY, HSQC, and HMBC experiments

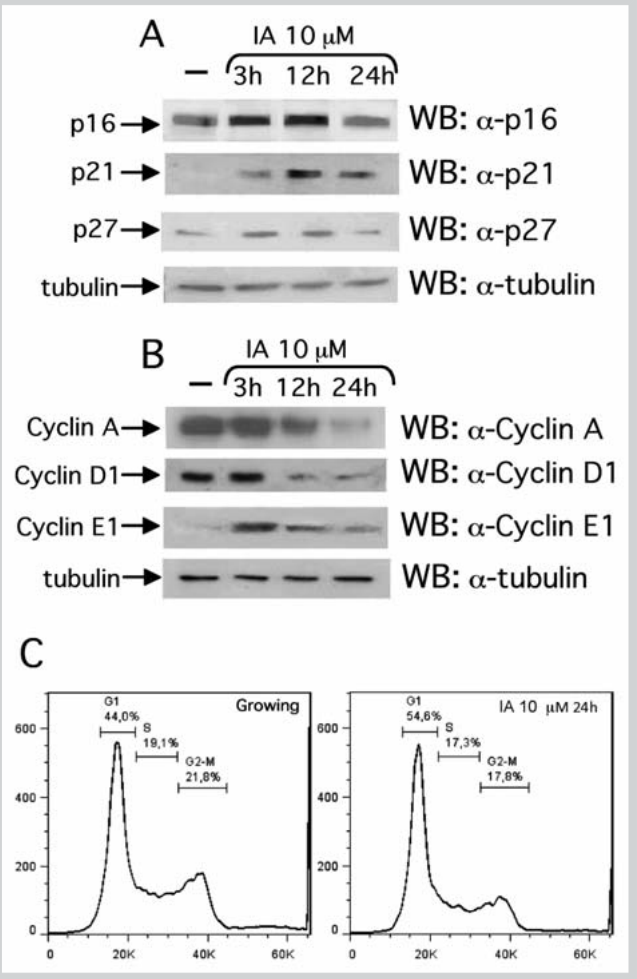

Fig. 2 CaLu-6 cells were exposed to 10 µM imbricatolic acid (IA) for the indicated times, and 40 µg of cell lysates were loaded on 12% SDS-PAGE. **A** The upregulation of $p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$, and $p16^{INK4a}$ and **B** degradation of cyclin A, cyclin D1, and cyclin E1 were immunodetected with specific antibodies. An anti-tubulin antibody was used as a control of protein loading. Cells were also exposed to 0.1% DMSO (–) for 12 h, as a control. **C** 5×10^6 cells were plated in 6-mm wells in the presence or absence of 10 µM imbricatolic acid (IA) for 24 h. Growing cells were incubated with 0.1% DMSO. Cells were resuspended in 300 µL of cold PBS and fixed with 1 mL of 70% ice-cold ethanol for 2 h. FACS analysis was performed as described in Materials and Methods by using a DAKA Cytomatio flow cytometer. Data were analyzed using Summit® 4.3 software. All the experiments were performed in triplicate.

Results and Discussion

Juniperus communis berries extracted with MeOH were subjected to Kupchan's methodology [11] to give *n*-hexane, $CHCl_3$, *n*-BuOH extracts, and an aqueous residue. The pure compounds were isolated, and their structures were determined by spectroscopic methods by comparison with the spectral data reported in the literature: *cis*-communic acid (**6**) [15], imbricatolic acid [15-hydroxy-labd-8(17)-en-19-oic acid] (**2**) [16], imbricatolal (**3**) [17], junicedric acid (**4**) [18], junicedral (**5**), agathadiol (**8**) [19], isoagatholal (**9**) [20], myrcecommunic acid (**10**) [21], sandarocopimaric acid (**11**) [22], isopimaric acid (**12**) [23], oplopanone (**16**) [24], and matairesinol (**17**) [25] (**Fig. 1**).

The new dihydrobenzofuran lignan glycoside, named juniperoside A (**1**) ($[\alpha]_D^{25}$ + 34.4), showed a pseudomolecular ion at *m/z* 493.2074 in its HRFABMS, and the presence of 25 carbon atoms in the ^{13}C NMR spectrum (**Table 2**) suggested the molecular formula $C_{25}H_{32}O_{10}$. In the ESI-MS (pos. ion mode) spectrum the pseudomolecular ion peak at *m/z* 515 $[M + Na]^+$ was also present. Its IR spectrum showed absorption bands at 3451 and 1660 cm^{-1} corresponding to hydroxyl and aromatic groups, respectively. The data from ^{1}H and ^{13}C NMR spectra of (**1**) were similar to those of clemastanin A [26], a glucoside isolated from *Clematis* species. Differences were detected in the sugar moiety. The ^{1}H NMR spectrum of juniperoside A showed three aromatic proton signals at δ_H 6.94, 7.06, and 7.08 which were assigned to three protons in a 1,2,4-trisubstituted benzene ring. Further two aromatic proton signals at δ_H 6.59 and 6.62 were assigned to two protons in a 1,3,4,5-tetrasubstituted benzene ring. The ^{1}H NMR spectrum also revealed proton signals due to two oxygenated methylene protons (δ_H 3.88 ovl/3.77 dd and 3.58 t), an oxygenated methine signal at δ_H 5.57 (d), and one methoxyl signal at δ_H 3.83 (s). By the aid of a ^{1}H-^{1}H COSY experiment, a propanol side chain was detected; a second spin system sequence evidenced oxygenated methylene protons (3.88/3.77) which were connected to a methine proton at δ_H 3.47. This last proton was also coupled with a proton signal at δ_H 5.57. In addition to ^{13}C NMR assignment, further HSQC experiment correlated the proton resonances of aglycone, with the relevant carbons indicating a dehydrodiconiferyl alcohol-type lignan. Significant HMBC correlations were reported in **Table 2**. The presence of a sugar moiety was deduced from the anomeric proton signal at δ_H 5.35 (br s) in the ^{1}H NMR spectrum. Starting from the anomeric proton signal, the proton resonances were assigned by ^{1}H-^{1}H COSY and TOCSY experiments. On

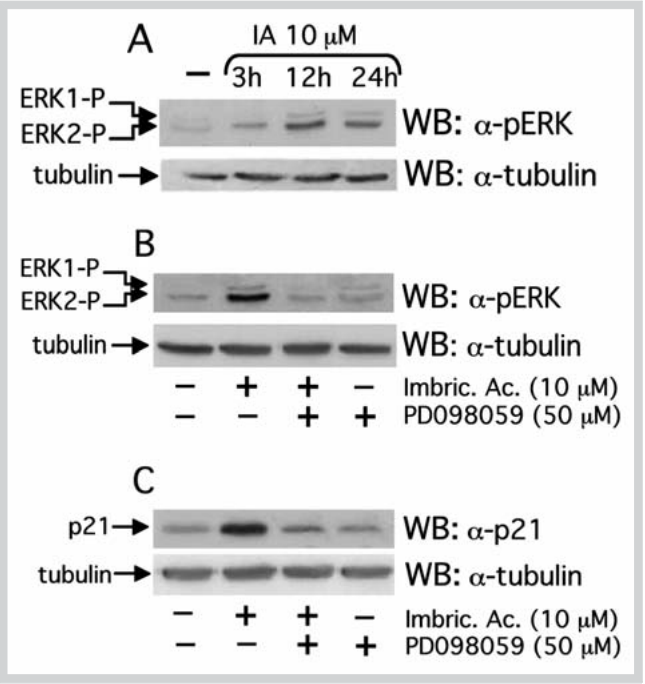

Fig. 3 Cell lysates were obtained from CaLu-6 cells exposed to 10 μM imbricatolic acid (IA) for the indicated times **A** or preincubated with PD098059 before the stimulation with 10 μM IA for 12 h (**B** and **C**). Forty micrograms of proteins were resolved by electrophoresis on 12% SDS-PAGE and transferred to a nitrocellulose membrane. ERKs phosphorylation was detected by using an anti-phosphoERK antibody (**A** and **B**). The arrows indicate the phosphorylated forms of $p44^{MAPK}$ (ERK1-P) and $p42^{MAPK}$ (ERK2-P). **C** $p21^{Cip1/Waf1/Sdi1}$ (p21) was immunodetected by using a specific antibody. An anti-tubulin antibody was used as a control of protein loading. Cells were also exposed to 0.1% DMSO (−) for 12 h. The experiments were performed in triplicate.

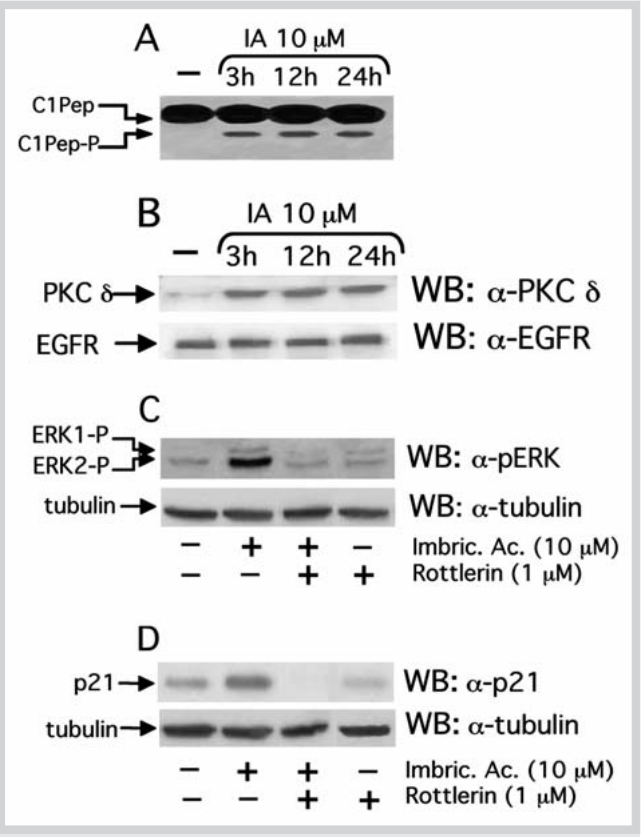

Fig. 4 **A** Assay of nonradioactive detection of PKC. 1×10^7 CaLu-6 cells were incubated with 10 μM imbricatolic acid (IA) for the indicated times or with 0.1% DMSO as a control (−). Cell lysates were purified on DEAE cellulose, and PKC assays were performed in triplicate as described in Materials and Methods. The reactions were stopped by heating to 95 °C for 10 min, and the samples were separated on a 0.8% agarose gel at 100 V for 15 min and photographed on a transilluminator. **B** Membranes were purified from CaLu-6 cells stimulated with 10 μM imbricatolic acid (IA) for the indicated times, and PKC*δ* was detected by a specific antibody. The same filter was incubated with anti-EGFR antibody as a control of protein loading. Cells were also exposed to 0.1% DMSO (−) for 12 h. Data is representative of three independent experiments. **C** and **D** Cellular extract were obtained from CaLu-6 cells stimulated for 12 h with 10 μM imbricatolic acid in the presence or absence of rottlerin. **C** Twenty micrograms of proteins were subjected to immunoblotting analysis with an anti-phosphoERK antibody (*α*-pERK). **D** Forty micrograms of proteins were electrophoresed on 12% SDS-PAGE, and the blot was incubated with an anti-$p21^{Cip1/Waf1/Sdi1}$ antibody (*α*-p21). The same filters were reprobed with an anti-tubulin antibody. The experiments were performed in triplicate.

acid hydrolysis with 2N CF_3CO_2H, **1** afforded rhamnose. The L configuration of rhamnose was assigned by GC analysis [12].

The connection between the rhamnopyranosyl unit to C-3′ of the aglycone was verified by the cross-peak between δ_H 5.35 (H-1″) and δ_C 146.0 (C-3′) in the HMBC experiment. HMBC cross-peaks also revealed the attachment of the methoxyl group at C-3 and the propanol side chain at C-1′ position. The stereochemistry between the hydroxymethyl and the aryl group was suggested to be *trans* by the ROESY experiment, by a coupling constant of H-7/H-8 (J = 5.7 Hz) as well as their chemical shifts. An intense ROE was observed between H-8/H-6′ (δ_H 3.47/6.62) and H-7/H_2-9 (δ_H 5.57/3.88–3.77), H-7/H-6 (δ_H 5.57/6.94), and H-7/H-2 (δ_H 5.57/7.06). Then the structure of juniperoside A **1** was determined to be 3-methoxy-3′,4,9,9′-tetrahydroxy-4′,7-epoxy-5′,8-lignan-3′-O-*α*-L-rhamnopyranoside.

Some labdane-type diterpenes have been shown to inhibit cell proliferation in several tumor cell lines which express functional p53 or that do not express p53 [8]. Therefore, we examined the effect of imbricatolic acid (IA) (**2**) on cell cycle inhibitory proteins $p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$, and $p16^{INK4a}$, as well as on cell cycle arrest, in p53-null human cancer CaLu-6 cells [27]. Cells were incubated with 10 μM IA for increasing times, and Western blot analysis showed a significant induction of these CKIs in a time-dependent manner with a maximal accumulation occurring after 12 h of treatment (**Fig. 2A**). We next evaluated the effect of IA on the protein level of cyclins. CKI of the Cip/Kip family can modulate the activities of cyclin D-, E-, and A-CDK complexes [9], which are involved in G1, G1/S, and S checkpoints, respectively. Labdane treatment of cells resulted in significant time-regulated decrease in the expression of cyclin A, D1, and E1 (**Fig. 2B**). Furthermore, DNA staining by propidium iodide showed that the exposure to 10 μM IA for 24 h induces the accumulation in G1 phase (**Fig. 2C**). We also analyzed the effects of junicedric acid (**4**) and agathadiol (**8**), which differ from IA for a carboxylic function on C15 (**4**) and for a primary alcoholic function on C19 and a double bond on C13–C14 (**8**). No effects of the two compounds on cell cycle at 10 μM were detected (data not shown).

In different cell types, the Ras/MAPK pathway mediates growth arrest by controlling cell cycle regulatory proteins [28–31]. Furthermore, in p53-null cells the accumulation of $p21^{Cip1/Waf1/Sdi1}$ is

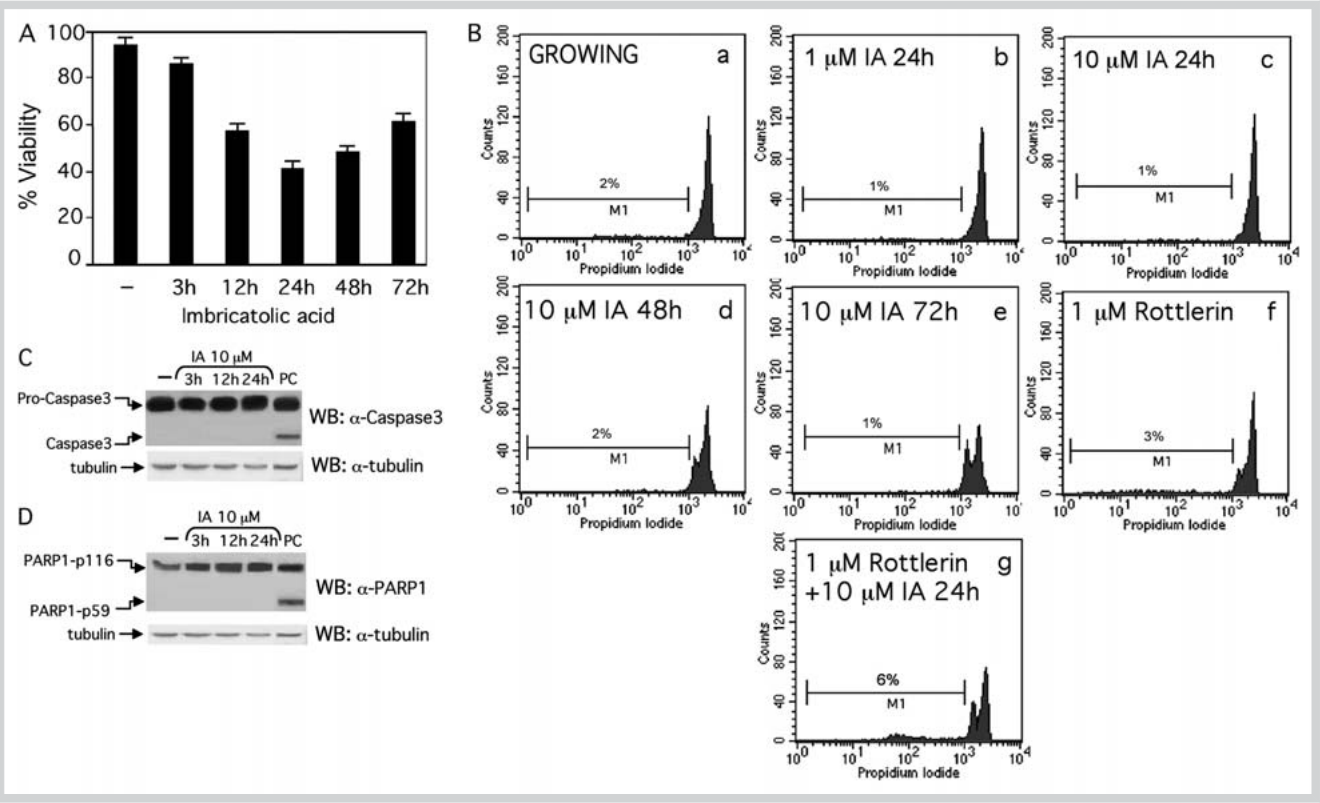

Fig. 5 **A** Cells were incubated in complete culture medium containing 10 μM imbricatolic acid or 0.1% DMSO (–) for the indicated times. MTT solution was added, and formazan crystals were then dissolved and measured at λ540 nm. Four independent experiments were performed. **B** Cells were incubated with 1 μM or 10 μM imbricatolic acid (IA) for the indicated times in the presence or absence of 1 μM rottlerin for 1 h. Growing cells were exposed to 0.1% DMSO as a control. Cells were incubated in a solution containing 50 μg/mL propidium iodide and analyzed by cytofluorimetry. The data are representative of at least three separate experiments of identical design. **C** and **D** CaLu-6 cells were exposed to 10 μM imbricatolic acid (IA) for the indicated times. Fifty micrograms of whole lysates were resolved on 10% SDS-PAGE and then incubated with specific antibodies against Caspase3 (**C**) or PARP1 (**D**). Cells were also exposed to etoposide as a positive control (PC) or to 0.1% DMSO (–) for 12 h. An anti-tubulin antibody was used as a control of protein loading. The experiments were performed in triplicate.

a consequence of the activation of Ras-ERK pathway [32]. Therefore, we investigated the molecular mechanisms underlying the upregulation of $p21^{Cip1/Waf1/Sdi1}$ by analyzing the ability of IA to activate ERKs. The incubation for increasing times with the labdane induced a sustained phosphorylation of ERKs (**Fig. 3A**) which was prevented by the MEK inhibitor PD098059 (**Fig. 3B**). We also observed that the preincubation with PD098059 prevents the IA-induced $p21^{Cip1/Waf1/Sdi1}$ accumulation (**Fig. 3C**), suggesting that this requires ERKs activation.

PKCδ is generally considered a growth inhibitor that can contribute to the p53-independent accumulation of $p21^{Cip1/Waf1/Sdi1}$ [33, 34]. We first investigated the ability of IA to activate PKC in CaLu-6 cells. **Fig. 4A** shows that PKC activity was detectable after 3 h and was sustained after 24 h of exposure to 10 μM IA. We next analyzed PKCδ activation by analyzing cellular partitioning of this isoenzyme [13]. In response to the labdane, PKCδ translocated into the membrane fraction, and a significant increase in the amount was observed within 3 h of treatment (**Fig. 4B**). We also observed that preincubation of CaLu-6 cells with 1 μM rottlerin, a selective inhibitor of PKCδ enzyme activity [35], caused a significant inhibition of IA-induced ERKs activation (**Fig. 4C**) and $p21^{Cip1/Waf1/Sdi1}$ accumulation (**Fig. 4D**). These results strongly suggest that, in CaLu-6 cells, IA induces PKCδ activation which is involved in the regulation of ERKs and, in turn, in the accumulation of a specific CKI.

To evaluate the effect of IA on cell viability of human CaLu-6 cells, we performed an MTT assay. The cells, exposed for different times with IA, showed a significant time-dependent inhibition of cell viability (**Fig. 5A**), as observed by a 89.8, 53.4, 41.4, 49.8, and 62.7% decrease after 3, 12, 24, 48, and 72 h, respectively. To test whether the decrease in cell growth was due to induction of apoptosis, we performed propidium iodide staining on CaLu-6 cells exposed for different times or concentrations to IA in the presence or absence of rottlerin. The results showed that the incubation with 1 μM or 10 μM IA for 24, 48, or 72 h (**Fig. 5B**, panels **a–e**) as well as the incubation with 1 μM rottlerin (**Fig. 5B**, panel **f**), or the pretreatment with rottlerin before IA stimulation for 24 h (**Fig. 5B**, panel **g**) did not induce any significant apoptosis. Furthermore, we performed Western blot analysis of PARP-1 and caspase3 by using specific antibodies. **Fig. 5C** shows that IA did not induce the cleavage of the 32 kDa precursor of caspase3 and, as a consequence, the 116 kDa PARP1 protein resulted not cleaved upon IA treatment (**Fig. 5D**). Taken together these data suggest that imbricatolic acid has not an apoptotic effect on CaLu-6 cells. The same results were obtained on the p53-null PC-3 cell line (data not shown).

In summary, we found that imbricatolic acid induces cell cycle arrest in CaLu-6 cells. A possible mechanism is: i) the accumulation of $p21^{Cip1/Waf1/Sdi1}$, mediated by PKCδ activation and ERKs phosphorylation; and ii) the decrease of cyclins A, D1, and E1 levels,

which are considered checkpoints of S, G1, and G1/S phases transition, respectively. Further studies are in progress to evaluate the role of $p27^{Kip1}$ and $p16^{INK4a}$ in the labdane-induced cell cycle arrest.

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Conflict of Interest

All authors declare no conflict of interest.

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