Dactylone Inhibits Epidermal Growth Factor–Induced Transformation and Phenotype Expression of Human Cancer Cells and Induces G_1-S Arrest and Apoptosis

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Abstract

The marine natural chamigrane-type sesquiterpenoid, dactylone, is closely related to secondary metabolites of some edible species of red algae. In the present study, the effect of dactylone was tested on the mouse skin epidermal JB6 P^+ Cl41 cell line and its stable transfectants as well as on several human tumor cell lines, including lung (H460), colon (HCT-116), and skin melanomas (SK-MEL-5 and SK-MEL-28). This natural product was effective at nontoxic doses as a cancer-preventive agent, which exerted its actions, at least in part, through the inhibition of cyclin D3 and Cdk4 expression and retinoblastoma tumor suppressor protein (Rb) phosphorylation. The inhibition of these cell cycle components was followed by cell cycle arrest at the G_1-S transition with subsequent p53-independent apoptosis. Therefore, these data showed that application of dactylone and related compounds may lead to decreased malignant cell transformation and/or decreased tumor cell proliferation. [Cancer Res 2007;67(12):5914–20]

Introduction

Consumption of various types of seafood has been suggested to be responsible for the low incidence of cancer in Japan and other countries whose inhabitants traditionally consume high levels of marine organisms. Many kinds of seafood are known to contain the low molecular weight natural products, which may be found only from these specific sources. However, a possible role of the secondary metabolites, besides fatty acids, from seafood (1) in this phenomenon has been insufficiently studied.

Halogenated chamigrane sesquiterpenoids represent a characteristic group of marine natural products that may be found only in red algae and mollusks feeding on them (2, 3). A variety of these secondary metabolites were identified in different species of algae belonging to the genus *Laurencia*, including those used as food condiments by the Japanese, Hawaiians, and other distinct populations. However, the anticancer properties of these compounds have not yet been examined.

Halogenated sesquiterpenoid dactylone [10-bromo-β-chamigren-4-one, IUPAC: (6*S*,10*R*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5,5]undec-2-en-4-one; Fig. 1*A*] was isolated from the sea hare *Aplysia dactylomela* along with two other sesquiterpenoids (4–7). The chemical structure of the dactylone (Fig. 1*A*) is closely related to the known sesquiterpenoids from *Laurencia* spp. (3, 8). Here, we report the cancer-preventive properties of dactylone, including some details regarding the molecular mechanism of this action.

Materials and Methods

Drugs and chemicals. Dactylone was isolated from the sea hare *A. dactylomela* as described previously (5). Minimum essential medium (MEM), DMEM, RPMI medium, and McCoy's medium were purchased from Life Technologies Invitrogen Corporation. Fetal bovine serum (FBS) was from Gemini Bio-Products; penicillin/streptomycin and gentamicin were from Bio-Whittaker; and L-glutamine was from Mediatech, Inc. Epidermal growth factor (EGF) was obtained from Collaborative Research. The luciferase assay substrate and Cell Titer 96 Aqueous One Solution Reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)] kit for the cell proliferation assay were from Promega. The mouse monoclonal IgG against p53, the specific p38 kinase inhibitor, SB202190, and specific c-Jun-NH_2-kinases inhibitors, SP600125 and 420116 peptide-type inhibitor, were from Oncogene Research Products. Phosphor-specific mitogen-activated protein (MAP) kinase antibodies against phosphorylated forms of p38 kinase, JNK1/2 and extracellular signal-regulated kinase 1/2 (ERK1/2) were from New England Biolabs. The phosphor-p53 antibody sampler kit was purchased from Cell Signaling Technology, Inc., and the Annexin V-FITC Apoptosis Detection kit was from Medical & Biological Laboratories.

Cell culture. The JB6 P^+ Cl41 mouse epidermal cell line and its stable transfectants were cultured as described previously (9). The human lung H460, colon HCT-116, and skin melanoma SK-MEL-28 and SK-MEL-5 cancer cell lines were obtained by American Type Culture Collection and were cultured in monolayers at 37°C and 5% CO_2 in RPMI, McCoy's, and MEM, respectively, containing 10% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. RSK2-deficient and wild-type RSK2 human lymphoblasts were cultured at 37°C with 5% CO_2 in RPMI medium containing 20% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Information regarding the genetic background of these cell lines is available online.[3]

Cell viability assay. The effect of dactylone on cell viability was evaluated using MTS reduction into its formazan product. The JB6 P^+ Cl41 mouse epidermal cell line and its stable transfectants, JB6 Cl41 dominant-negative mutant (DNM)-JNK1, DNM-p38, DNM-ERK2, and RSK2-deficient and wild-type RSK2 human cells, and human lung cancer H460 cells were cultured for 12 h in 96-well plates (6,000 per well). The media were then replaced with corresponding media containing 0.1% FBS and dactylone at various concentrations in a total volume of 0.1 mL and the cells were incubated for 22 h. Then, 20 μL of the MTS reagent were added into each well and MTS reduction was measured 2 h later spectrophotometrically at 492 and 690 nm as background using the Multiskan MS microplate reader (Labsystems).

Anchorage-independent transformation or phenotype expression assay. The cancer-preventive and/or therapeutic effects of dactylone were evaluated using an anchorage-independent neoplastic transformation or

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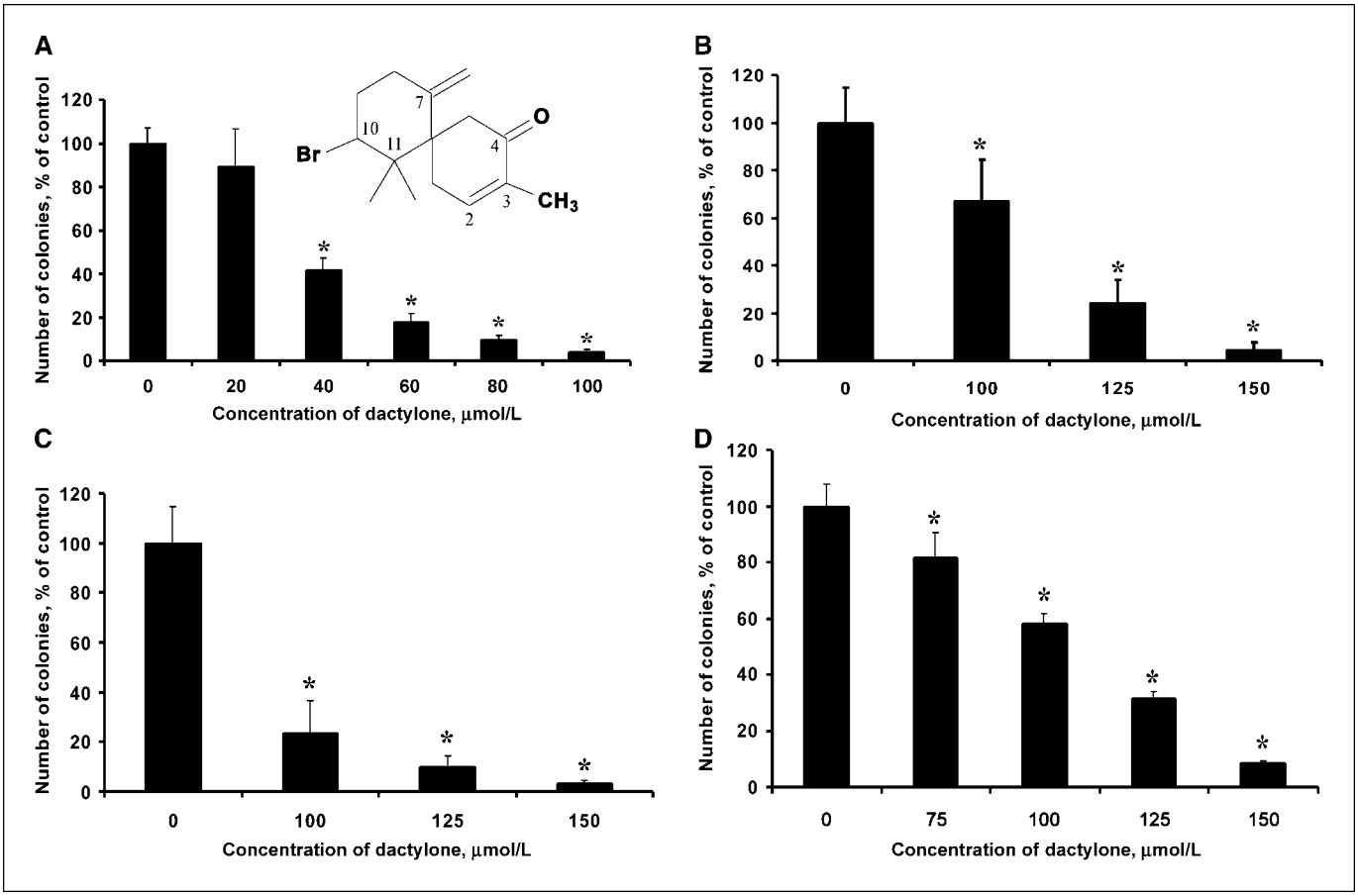

Figure 1. Dactylone suppresses JB6 cell transformation and human tumor cell phenotype expression. *A,* the chemical structure of dactylone. The experiments were carried out as described in Materials and Methods. At nontoxic concentrations, dactylone suppressed malignant transformation of JB6 P^+ Cl41 cells (*A*) and phenotype expression of human lung cancer H460 cells (*B*), human colon cancer HCT-116 cells (*C*), and human melanoma SK-MEL-5 cells (*D*) in soft agar. Results are shown as a percentage of untreated control cells. *Columns,* mean from six samples of two independent experiments; *bars,* SD. *, $P < 0.05$, significant decrease in colony formation induced by dactylone compared with untreated control cells.

phenotype expression assay. For evaluation of the anticancer effects of dactylone in various human cancer cell lines using a phenotype expression assay, no additional stimulus was required. EGF (10 ng/mL) was used for stimulating neoplastic transformation of JB6 P^+ Cl41 cells. The assay was carried out in six-well tissue culture plates. Human H460, HCT-116, SK-MEL-5, or mouse JB6 P^+ Cl41 cells (8×10^3/mL) were treated with various concentrations of dactylone in 1 mL of 0.33% basal medium Eagle (BME) agar containing 10% FBS over 3 mL of 0.5% BME agar containing 10% FBS and various concentration of dactylone. The cultures were maintained in a 37°C, 5% CO_2 incubator for 1 week. Cell colonies were then scored using a LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics). Data for $INCC_{50}$ (inhibition of the number of colonies C_{50}) of dactylone were obtained from anchorage-independent transformation or phenotype expression assays, using regressions that were built by the computer program Statistica 6.0 (StatSoft, Inc.).

Cell cycle assay. Cell cycle arrest at G_1-S phase induced by dactylone was studied by flow cytometry. JB6 P^+ Cl41 and SK-MEL-28 cells were plated (8×10^5/10-cm dish) in 5% (JB6 cells) or 10% (SK-MEL-28 cells) FBS/MEM and allowed to attach overnight. Then, the cells were starved in 0.1% FBS/MEM for 36 h and followed by incubation for 1 h with various concentrations of dactylone. Five percent (JB6 cells) or 10% FBS (SK-MEL-28 cells) were added to each dish, except control cells, and cells were incubated for another 16 h. After treatment with dactylone, cells were washed with PBS and harvested with 0.025% trypsin in 5 mmol/L EDTA in PBS. Trypsinization was stopped by adding 2 mL of 5% FBS in PBS. The obtained suspension was centrifuged at 1,000 rpm (170 rcf) for 5 min, and cells were resuspended in 0.4 mL of PBS. Then, 1 mL of ice-cold ethanol was added and cells were fixed at −20°C for at least 2 h. After washing with PBS, cells were stained with 20 μg/mL of propidium iodide and RNase (200 μg/mL) for 30 min at room temperature in the dark. DNA content was analyzed by a Becton Dickinson FACSCalibur Flow Cytometer (BD Biosciences). The population of cells in each cell cycle phase was determined using ModFIT LT software (Verity Software House, Inc.).

Western blot analysis. The effect of dactylone on the intracellular levels of phosphorylated or nonphosphorylated kinases and selected proteins in JB6 Cl41 and human tumor cells was studied using Western blot analysis.

Phosphorylation of ERKs, JNKs, and p38. JB6 Cl41 cells were plated (1×10^6/10-cm dish) in 5% FBS/MEM and allowed to attach overnight. Then, the cells were starved in 0.1% FBS/MEM for 24 h and incubated with 100 μmol/L dactylone for various times or with one concentration of dactylone for 15 min if phosphorylation of ERKs or p38 kinase was studied or for 30 min if phosphorylation of JNKs was studied. Then, cells were washed with PBS and disrupted with 200 μL radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L Na_3VO_4, 1 mmol/L aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). The samples were sonicated and centrifuged at 15,000 × *g* for 15 min. The quantity of protein was measured by the Bradford method (10) and normalized against controls. The samples were heated to 95°C for 5 min, cooled on ice, and centrifuged at 15,000 × *g* for 5 min. The samples (30 μg) were then resolved by 8% SDS-PAGE. Immunoblotting analysis of

phosphorylated ERK1/2, JNK1/2, or p38 kinases was carried out using specific antibodies against phosphorylated sites of ERK1/2 (Thr^{202}/Tyr^{204}), JNK1/2 (Thr^{183}/Tyr^{185}), or p38 (Thr^{180}/Tyr^{182}), respectively.

Determination of intracellular levels of cyclin D3, Cdk4, or phosphorylated Rb protein. JB6 Cl41 cells were plated (1×10^6/10-cm dish) in 5% (JB6 Cl41 cells) or 10% FBS/MEM (SK-MEL-28 cells) or 10% FBS/RPMI (H460 cells) and allowed to attach overnight. Then, the cells were starved in 0.1% FBS/MEM (JB6 Cl41 or SK-MEL-28 cells) or 0.1% FBS/RPMI (H460 cells) for 36 h. Human RSK2-deficient ($RSK2^-$) or wild-type RSK2 cells were plated (1×10^6/10-cm dish) in 10% FBS/RPMI and then starved in 0.1% FBS/RPMI for 36 h. Subsequently, the cells were incubated with various concentrations of dactylone for 1 h, and then 5% FBS (JB6 Cl41 cells) or 10% FBS (other cells) was added to each dish except control cells, and the cells were incubated for another 24 h. After incubation with dactylone, cells were washed with PBS and disrupted as described above. Then, the samples (30 μg) were resolved by 8% (12% for cyclin D3 protein or Cdk4) SDS-PAGE. Immunoblotting analysis of cyclin D3 protein, Cdk4, or phosphorylated Rb tumor-suppressor protein was carried out using specific antibodies against cyclin D3 protein or Cdk4, or against phosphorylated sites of the Rb protein (Ser^{780}, Ser^{795}, $Ser^{807/811}$), respectively.

Assessment of the effect of dactylone on p53, activator protein, and nuclear factor-κB–dependent transcriptional activities. The effect of dactylone on p53-, AP-1– or nuclear factor-κB (NF-κB)–dependent transcriptional activity in mouse JB6 Cl41 cells was evaluated using the luciferase method. Viable JB6 Cl41 PG-13 (p53), AP-1, or NF-κB cells (6×10^3) suspended in 100 μL 5% FBS/MEM were added into each well of a 96-well plate. Plates were incubated for 24 h and then treated with various concentrations of dactylone in 100 μL of 0.1% FBS/MEM. After incubation with dactylone for 24 h, the cells were disrupted for 1 h at room temperature with 100 μL/well of lysis buffer [0.1 mol/L potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mmol/L DTT, 2 mmol/L EDTA]. Then, 30 μL of lysate from each well were transferred into a plate for luminescent analysis and luciferase activity was measured using 100 μL/well of the luciferase assay buffer [1 mmol/L D-luciferin (pH 6.1–6.5), 40 mmol/L Tricin, 2.14 mmol/L magnesium carbonate hydroxide pentahydrate $(MgCO_3)_4 \times Mg(OH)_2 \times 5H_2O$, 5.34 mmol/L $MgSO_4 \times 7H_2O$, 66.6 mmol/L DTT, 1.06 mmol/L ATP, 0.54 mmol/L CoA, and 0.2 mmol/L EDTA (pH 7.8)] and the Luminoscan Ascent Type 392 microplate reader (Labsystems).

Apoptosis assessed by flow cytometry. The induction of early and late apoptosis by dactylone was analyzed by flow cytometry using the Becton Dickinson FACSCalibur Flow Cytometer (BD Biosciences). JB6 P^+ Cl41 or SK-MEL-28 cells (3×10^5 per dish), were grown in 6-cm dishes for 12 h in 5% FBS/MEM (JB6 P^+ Cl41) or 10% FBS/MEM (SK-MEL-28 cells). Then, cells were starved in 0.1% FBS/MEM for 12 h (JB6 P^+ Cl41 cells) or 24 h (SK-MEL-28 cells). Cells were then treated with dactylone in 0.1% FBS/medium for 72 h (JB6 P^+ Cl41 cells) or 24 h (SK-MEL-28 cells). After treatment with dactylone, the medium was collected and attached cells were harvested with 0.025% trypsin in 5 mmol/L EDTA in PBS. Trypsinization was stopped by adding 2 mL of 5% FBS in PBS. Cells were washed by centrifugation at 1,000 rpm (170 rcf) for 5 min and processed for detection of early and late apoptosis using Annexin V-FITC and propidium iodide staining according to the manufacturer's protocol. In brief, cells (1×10^5–5×10^5) were collected after centrifugation, and resuspended in 500 μL of 1× binding buffer (Annexin V-FITC Apoptosis Detection kit, Medical & Biological Laboratories). Then, 5 μL of Annexin V-FITC and 5 μL of propidium iodide were added, and the cells were incubated in the dark at room temperature for 5 min and analyzed by flow cytometry.

Results

Cytotoxic and cancer-preventive properties. The MTS method was applied to examine the potential cytotoxicity of dactylone. Dactylone was shown to be nontoxic against mouse epidermal JB6 P^+ Cl41 cells and human lung cancer cells H460 at doses of <150 and 200 μmol/L, respectively. To determine the effect of dactylone on cell transformation or phenotype expression, which are shown by colony formation of JB6 P^+ Cl41 or various human tumor cells, we used the standard anchorage-independent malignant transformation or phenotype expression assay (soft agar; refs. 11–20). To assess whether dactylone could prevent tumor promoter–induced neoplastic transformation of JB6 P+ Cl41 cells, we used EGF (10 ng/mL) as a promoter of colony formation. The results showed that dactylone exhibited cancer-preventive properties at noncytotoxic doses. Specifically, a 50% inhibition of EGF-induced colony formation ($INCC_{50}$) by dactylone was

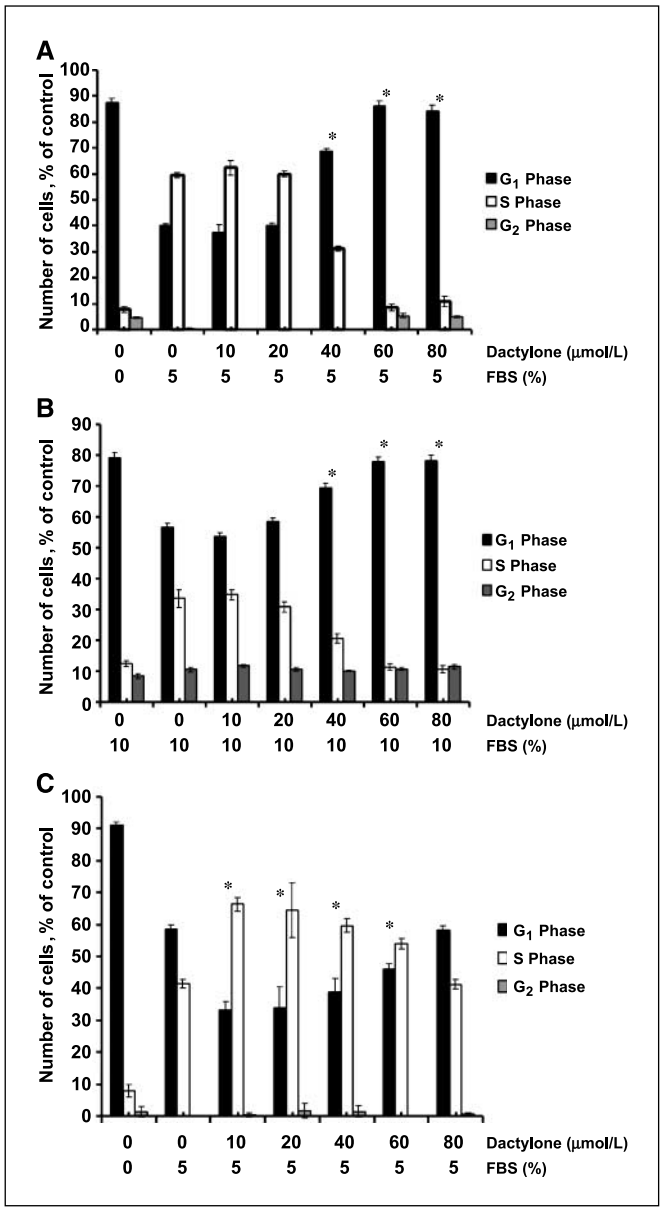

Figure 2. Dactylone induces cell cycle arrest at the G_1-S transition in JB6 P^+ Cl41 (*A*) and human skin melanoma SK-MEL-28 cells (*B*), but not in JB6 Cl41 DNM-ERK (*C*) cells. Cell cycle arrest in G_1-S phase induced by dactylone was studied by flow cytometry as described in Materials and Methods. The effects of dactylone on cell cycle progression are presented as a percentage of dactylone-treated cells in G_1, S, or G_2 phases of cell cycle compared with untreated control cells. *Columns,* means from four samples of two independent experiments; *bars,* SD. *, $P < 0.05$, significant increase in percentage of cells accumulated at the indicated phase induced by dactylone compared with untreated control cells.

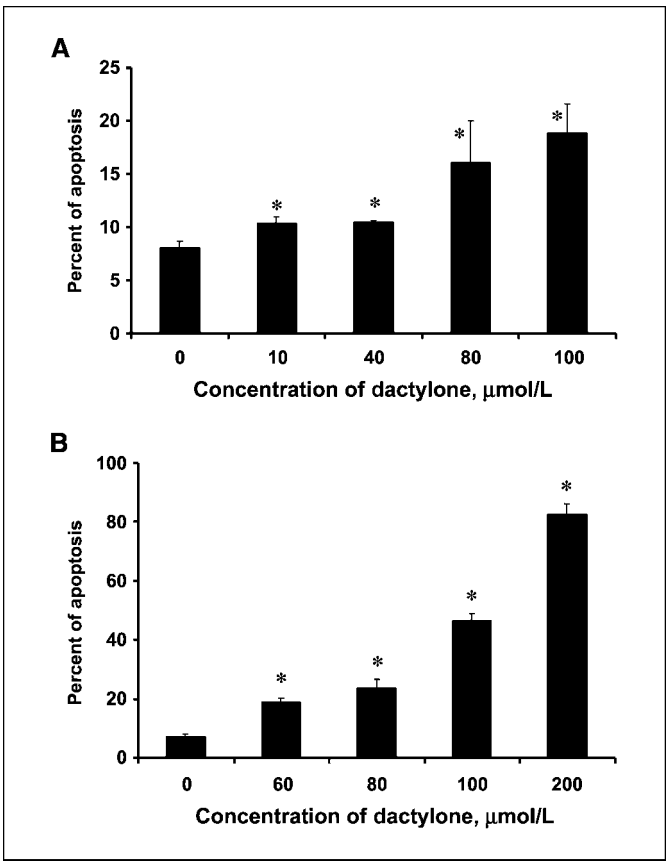

Figure 3. Dactylone induces apoptosis in JB6 P^+ Cl41 (*A*) or human skin melanoma SK-MEL-28 cells (*B*). The induction of apoptosis by dactylone in JB6 P^+ Cl41 or human melanoma (SK-MEL-28) cells was analyzed by flow cytometry using the Becton Dickinson FACSCalibur Flow Cytometer, as described in Materials and Methods. *Columns,* mean from four samples of two independent experiments; *bars,* SD. *, $P < 0.05$, significant increase in apoptosis induced by dactylone compared with untreated control cells.

achieved at a concentration of 45.4 μmol/L (Fig. 1*A*). Similar effects were observed for suppression of phenotype expression of several human tumor cell lines. For example, the $INCC_{50}$ for inhibition values of phenotype expression of lung cancer H460 cells (Fig. 1*B*), colon tumor HCT-116 cells (Fig. 1*C*), and skin melanoma SK-MEL-5 cells (Fig. 1*D*) were 92.4, 70.5, and 99.2 μmol/L, respectively. This suggested that dactylone suppressed phenotype expression of these cancers, and at least one of them, H460, at noncytotoxic doses.

Cell cycle analysis. Next, we evaluated probable mechanisms to explain the anticancer action of dactylone, and, in particular, to evaluate its effect on cell cycle progression. JB6 P^+ Cl41 (Fig. 2*A*), SK-MEL-28 (Fig. 2*B*), or JB6 DNM-ERK (Fig. 2*C*) cells were synchronized at G_0 by serum deprivation, restimulated with FBS, and then treated for 16 h with increasing concentrations of dactylone. Results of flow cytometry analysis showed that dactylone induced cell cycle arrest in both JB6 P^+ Cl41 and SK-MEL-28 cells at the G_1-S transition similar to serum deprivation. Dactylone at 40 μmol/L significantly prevented the JB6 P^+ Cl41 cell transition from G_1 to the S phase; at 60 μmol/L, it completely blocked cell cycle progression at the G_1-S (Fig. 2*A*). In contrast, in DNM-ERK cells (Fig. 2*C*), dactylone (10–60 μmol/L) intensified the transition of cells from G_1 to S phase. Based on these data, we suggest that the effect of this compound on the cell cycle progression may be mediated, at least in part, by ERKs.

Drug-induced apoptosis. The cell cycle data suggested that dactylone might induce programmed cell death or apoptosis.

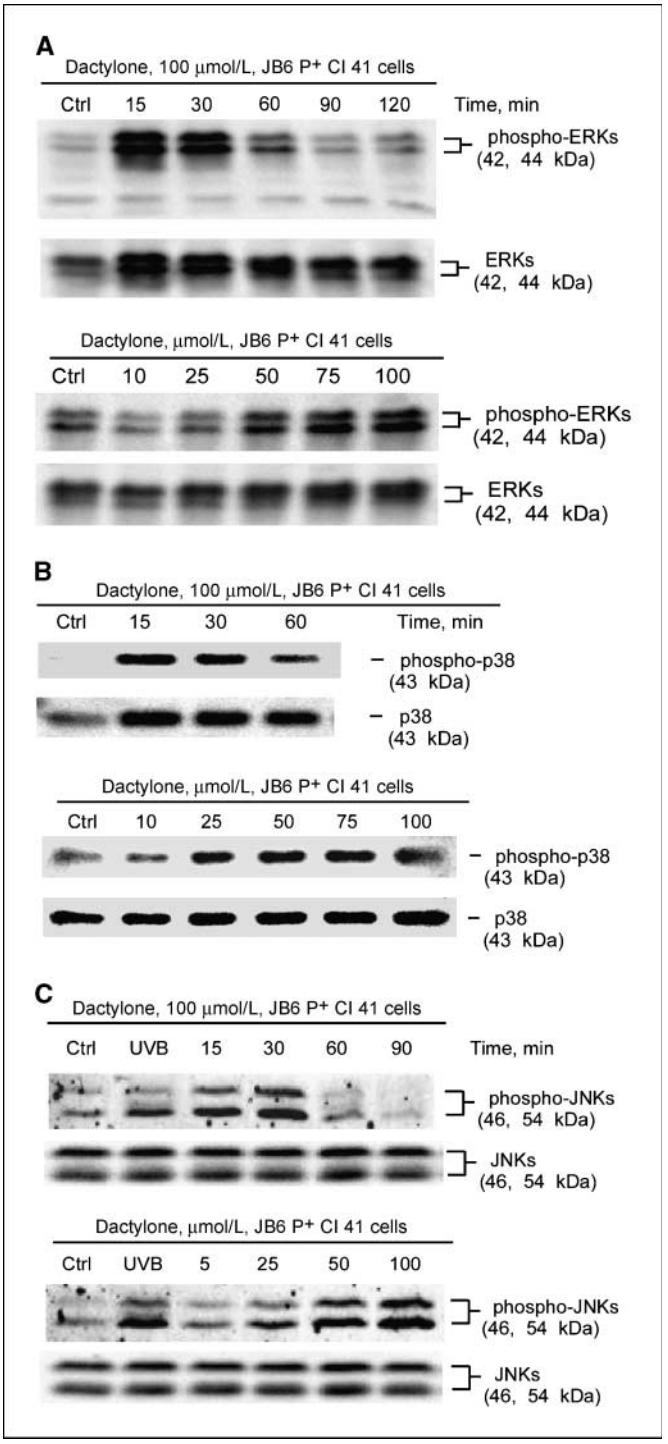

Figure 4. Dactylone has differential effects on the phosphorylation of ERKs, JNKs, and p38 kinase in JB6 Cl41 cells. The time- and dose-dependent effects of dactylone on the intracellular levels of phosphorylated or nonphosphorylated ERKs (*A*), p38 (*B*), or JNKs (*C*) in JB6 P^+ Cl41 cells was studied using Western blot analysis as described in Materials and Methods. Dactylone induced phosphorylation of ERKs, p38 kinase, and JNKs at 50 μmol/L in JB6 P^+ Cl41 cells. The optimal time for induction of ERKs and p38 phosphorylation was 15 min, whereas the optimal time of JNKs phosphorylation was ~30 min. Representative blots of three independent experiments for each kinase.

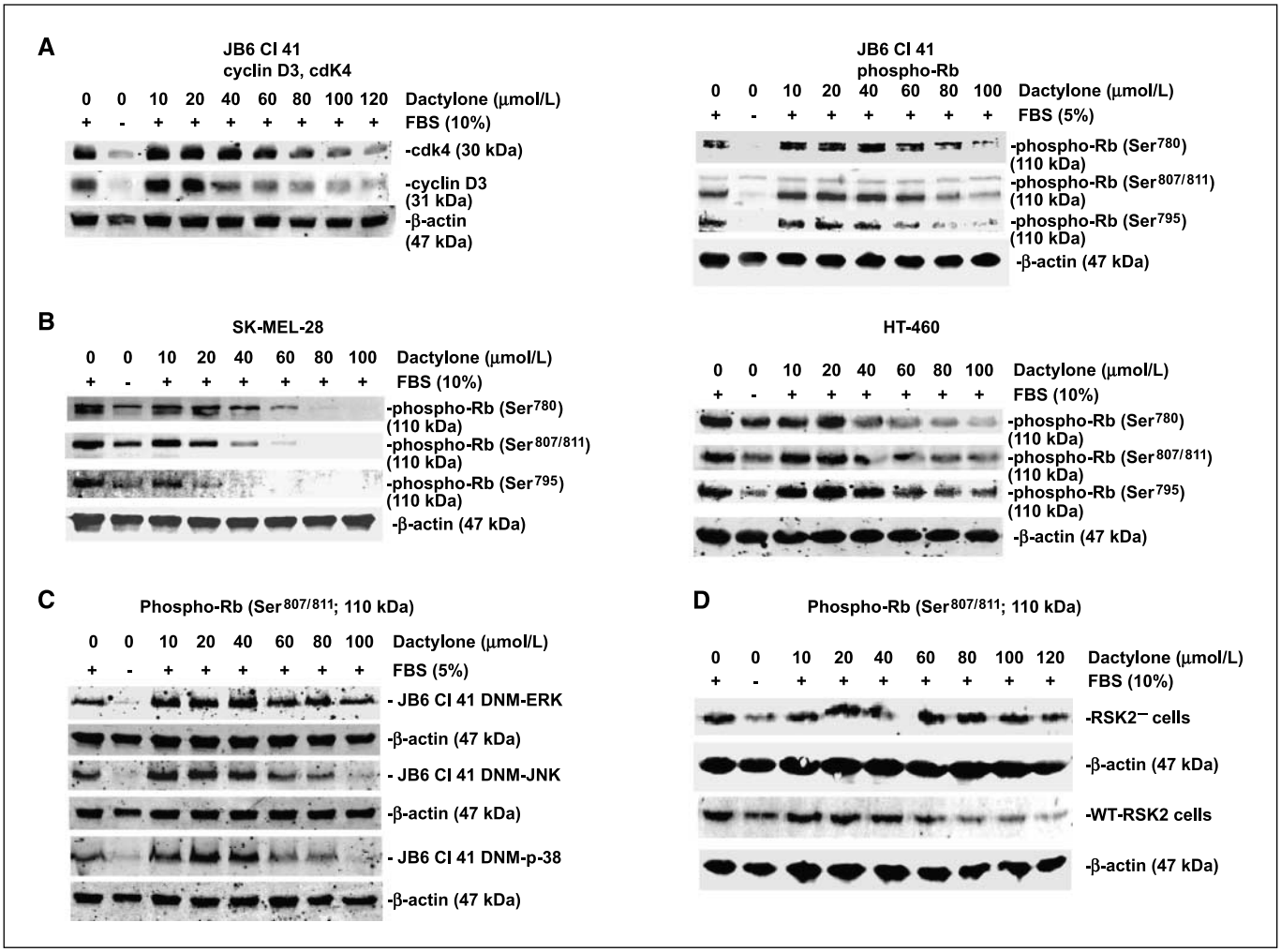

Figure 5. Dactylone suppresses cyclin D3 and Cdk4 protein levels and phosphorylation of the Rb protein. The dose-dependent effects of dactylone on the intracellular levels of cyclin D3, Cdk4, and phosphorylation of Rb in JB6 P^+ Cl41 cells (*A*), melanoma SK-MEL-28 and H460 lung cancer cells (*B*), JB6 Cl41 DNM cells (*C*), and RSK2-deficient and wild-type RSK2 cells (*D*) were studied using Western blot analysis, as described in Materials and Methods. Dactylone treatment decreased expression of cyclin D3 and Cdk4 (*A*) and inhibited phosphorylation of the Rb protein of JB6 P^+ Cl41 cells at Ser^{780}, $Ser^{807/811}$, and Ser^{795} (*A*) in a dose-dependent manner. The same inhibition was observed in human melanoma SK-MEL-28 and lung cancer H460 cells (*B*). Interestingly, dactylone was significantly less effective in suppressing Rb phosphorylation in JB6 Cl41 ERK2 DNM cells compared with p38 DNM or JNK1 DNM JB6 Cl41 cells (*C*), indicating the important role of ERKs in mediating the dactylone effect. This effect was also significantly diminished in $RSK2^-$ (Coffin-Lowry Syndrome) patient cells compared with wild-type RSK2 cells (*D*). Representative blot from three independent experiments for each protein.

Induction of apoptosis by dactylone was evaluated by flow cytometry using Annexin V–FITC and propidium iodide staining. We found that dactylone induced a dose-dependent apoptosis of JB6 P^+ Cl41 (Fig. 3*A*) at nontoxic concentrations but also in human tumor melanoma (SK-MEL-28) cells (Fig. 3*B*). All these data supported the idea that the cancer-preventive action of dactylone may be explained, at least in part, by its ability to inhibit the cell transition from G_1 to S phase and induce apoptosis.

Effect of dactylone on the phosphorylation of key signaling proteins. Several studies have shown earlier that ERK phosphorylation and activation can induce cell cycle arrest (21–23). In our study, we showed that dactylone (50 μmol/L) induces phosphorylation of all the major MAP kinases, including ERKs (Fig. 4*A*), p38 (Fig. 4*B*), and JNKs (Fig. 4*C*) in JB6 P^+ Cl41 cells. ERKs and p38 phosphorylation was induced rapidly by 100 μmol/L dactylone (maximal by 15 min), whereas JNK phosphorylation was maximally induced by ~30 min. These data were in agreement with our preliminary findings, which showed that dactylone may stimulate intracellular processes such as cell cycle arrest mediated, at least in part, through ERKs.

Based on the results of the cell cycle assay, we also studied the effect of various doses of dactylone on the expression of cyclin D3, Cdk4, and the phosphorylation of the Rb tumor suppressor protein, which regulate progression through the G_1-S checkpoint of the cell cycle (Fig. 5). Results indicated that dactylone treatment decreased expression of cyclin D3 and Cdk4 in JB6 P^+ Cl41 cells (Fig. 5*A*). Dactylone also suppressed phosphorylation of the Rb protein of JB6 P^+ Cl41 cells at Ser^{780}, $Ser^{807/811}$, and Ser^{795} (Fig. 5*A*) in a dose-dependent manner. Similar inhibitory effects by dactylone were also observed in the human tumor SK-MEL-28 and H460 cell lines (Fig. 5*B*).

Additional evidence supporting an active role of the ERK signaling pathway in mediating the dactylone-induced inhibition of Rb phosphorylation with subsequent cell cycle arrest at the G_1-S transition was obtained comparing the effects of dactylone on JB6 P^+ Cl41 wild-type, DNM-ERK2, DMN-p38, and DNM-JNK1 cells

(Fig. 5*C*). Interestingly, at concentrations up to 100 μmol/L, dactylone had no effect on phosphorylation of Rb in DNM-ERK2 cells. In contrast, Rb phosphorylation was suppressed by dactylone in DNM-p38 and DNM-JNK1 cells. The inhibitory effect of dactylone was also significantly diminished in RSK2^{-} (Coffin Lowry Syndrome patient) cells compared with wild-type RSK2 cells (Fig. 5*D*). RSK2 is a key downstream target of the ERK signaling pathway (24).

Influence of dactylone on p53-dependent transcriptional activity. The effect of dactylone on p53-dependent transcriptional activity was assessed with JB6 P^{+} Cl41 cell lines stably expressing a luciferase reporter gene controlled by a p53 DNA-binding sequence. We found that dactylone at a nontoxic concentration (100 μmol/L) inhibited p53-dependent transcriptional activity in JB6 P^{+} Cl41 cells by ~2-fold (Fig. 6*A*). The significance of the decreased p53 nuclear factor–dependent transcriptional activation induced by dactylone was verified using nonparametric statistical method Mann-Whitney *U* test (computer program Statistica 6.0, StatSoft). The tumor-suppressor p53 protein plays a critical role in apoptosis and an increase of p53 protein production followed by apoptosis is a common characteristic effect of cancer-preventive agents such as resveratrol (25), black tea extract (26), and many others. However, in our case, we did not observe an increase in p53 protein levels, although apoptosis was induced by dactylone.

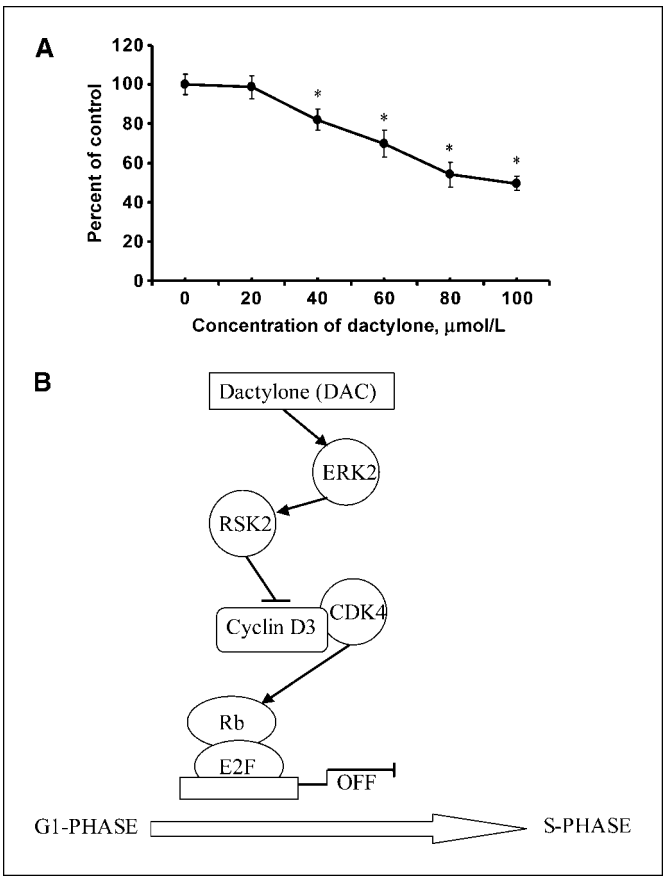

Figure 6. The effect of dactylone on p53-dependent transcriptional activity. *A,* the effect of dactylone on p53-dependent transcriptional activation in mouse JB6 Cl41 cells was evaluated using the luciferase reporter method, as described in Materials and Methods. Data represent the percentage of dactylone-treated p53-dependent transcriptional activation relative to untreated control cells. *Points,* mean from 10 samples of two independent experiments; *bars,* SD. *, $P < 0.05$, significant decrease or increase in p53 transcriptional activation induced by dactylone compared with untreated control cells. *B,* hypothetical scheme of the mechanism of the antitumor action of dactylone.

Discussion

Our data showed that dactylone is representative of a new group of natural cancer-preventive agents. To the best our knowledge, this is the first marine terpenoid metabolite used at noncytotoxic doses that inhibited cell transformation of normal mouse cells as well as suppressed the phenotype expression of various human cancer cell lines. Further details regarding a probable molecular mechanism of action were also elucidated. Results reported here showed that dactylone induced G_1-S cell progression arrest and apoptosis of tumor cells; decreased Rb protein phosphorylation at Ser^{795}, Ser^{780}, and Ser$^{807/811}$ sites; and inhibited expression of cyclin D3, Cdk4. Active, hypophosphorylated Rb is a potent negative regulator of cell cycle progression from G_1 into and through S phase (27–30). A possible mechanism of dactylone antitumor action is shown in Fig. 6. Several other previously reported cancer-preventive agents seem to act in a manner similar to dactylone. For example, like dactylone, inositol hexaphosphate, a dietary constituent found in rice, was reported to decrease cyclin-dependent kinase (Cdk) 4 and cyclin D1 protein levels and also showed an inhibitory effect on Rb phosphorylation at Ser^{780}, Ser^{807}, and Ser^{811}, causing G_1 arrest and apoptotic death of human prostate carcinoma LNCaP cells (31). However, in contrast to many proapoptotic drugs, dactylone did not increase p53 transcriptional activity. Similar cases have been previously reported in the scientific literature. For example, in PC-3 cells, indole-3-carbinol from fruit and vegetable diets inhibited Rb phosphorylation and induced G_1 cell arrest leading to apoptosis independently of the p53 responsible element (32). Flavone, a parent compound of dietary flavonoids, inhibited proliferation, migration, and capillary tube formation of human endothelial cells through the down-regulation of the hyperphosphorylated form of the Rb protein without an effect on the expression of p53 (33). However, the significance of the dactylone-associated decrease in p53 transcriptional activity as it related to the observed cancer-preventive effects of dactylone remains unclear. Similar to many other anticancer agents, the action of dactylone may be mediated at least partially through the MAP kinase signaling pathways. Actually, dactylone activates phosphorylation of all the primary MAP kinases. However, some of our results, including the absence of G_1-S transition arrest and the lack of inhibition of Rb protein phosphorylation in JB6 Cl41 ERK2 DNM cells, support the idea that ERKs play one of the main roles in the cellular response to dactylone application. Although this role also requires further investigation, other reports indicated that ERK1/2 and p38 may cooperate to induce G_1 cell cycle arrest (34).

All these results, as well as results from others, showed that marine chamigrane sesquiterpenoids (exemplified by dactylone), constituents of some exotic kinds of seafood, represent a prospective group of natural products as cancer-preventive and antitumor agents. Indeed, some marine brominated chamigrane sesquiterpenoids isolated from *Laurencia* spp. were reported (35, 36) to produce a novel profile of differential cytotoxicity in the National Cancer Institute *in vitro* screening system and were more cytotoxic to certain cell lines in the colon cancer subpanel at concentrations 10- to 100-fold lower than the average cytotoxic concentration observed in other tumor subpanels. Quite possibly, similar effects such as prevention of cell transformation,

suppression of tumor cell proliferation, or cell cycle arrest followed by apoptosis might be observed with the dietary use of some red algae. These actions may be accompanied by some other useful effects of the halogenated sesquiterpenoids such as a potent antibacterial activity against antibiotic-resistant bacteria and inhibition of *Mycobacterium tuberculosis* (37–41), antiviral activity against herpes simplex virus-1 (42), and anthelmintic properties (43) previously indicated for these types of natural products. These effects may explain, in part, some of the useful properties attributed to seafood gleaned from public opinion.

Taking into account that the majority of neoplasms seem to have aberrations in the retinoblastoma pathways due to hyperactivation of Cdks (44), the observation that dactylone might be a new structural type of intracellular inhibitor of cyclin D3, Cdk4, and phosphorylation of the retinoblastoma tumor-suppressor protein (Rb) is interesting. Therefore, the further search for similar inhibitors among natural products of this chemical series and their synthetic analogues may lead to the creation of new highly active molecular tools for anticancer molecular studies.

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References

1. Sauer LA, Dauchy RT, Blask DE. Mechanism for the antitumor and anticachectic effects of n-3 fatty acids. Cancer Res 2000;60:5289–95.
2. Martin JD, Darias J. Algal sesquiterpenoids. In: Scheuer PJ, Darias J, editors. Marine natural products, chemical and biological perspectives. New York: Academic Press; 1978. p. 125–73.
3. Fraga BM. Natural sesquiterpenoids. Nat Prod Rep 2005;22:465–86.
4. Lyakhova EG, Fedorov SN, Shubina LK. Chemical properties of marine terpenoids I. Some reactions of (6*S*,10*R*)-10-bromo-3,11,11-trimethyl-7-methylidenespiro[5,5] undec-2-en-4-one, a sesquiterpenoid from the sea hare, *Aplysia dactylomela*. Russ Chem Bull 2003;52:1022–6.
5. Fedorov SN, Reshetnyak MV, Schedrin AP. New halogenated chamigrane sesquiterpenoid from the mollusk, *Aplysia* sp. Structure and absolute configuration. Dok1 AN SSSR 1989;305:877–9.
6. Fedorov SN, Shubina LK, Kalinovsky AI, et al. Structure and absolute configuration of a new rearranged chamigrane-type sesquiterpenoid from the sea hare *Aplysia* sp. Tetrahedron Lett 2000;41:1979–82.
7. Fedorov SN, Radchenko OS, Shubina LK, et al. Aplydactone, a new sesquiterpenoid with an unprecedented carbon skeleton from the sea hare *Aplysia dactylomela*, and its cargill-like rearrangement. J Am Chem Soc 2001;123:504–5.
8. Suzuki M, Kurosawa E, Irie T. Three new sesquiterpenoids containing bromine, minor constituents of *Laurencia glandulifera* Kutzing. Tetrahedron Lett 1974;15:821–4.
9. Fedorov SN, Bode AM, Stonik VA, et al. Marine alkaloid polycarpine and its synthetic derivative dimethylpolycarpine induce apoptosis in JB6 cells through p53- and caspase 3-dependent pathways. Pharm Res 2004;21:2307–19.
10. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
11. Colburn NH, Former BF, Nelson KA, et al. Tumour promoter induces anchorage independence irreversibly. Nature 1979;281:589–91.
12. Colburn NH, Wendel EJ, Abruzzo G. Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: mitogen-resistant variants are sensitive to promotion. Proc Natl Acad Sci U S A 1981;78:6912–6.
13. Dong Z, Cmarik JL, Wendel EJ, et al. Differential transformation efficiency but not AP-1 induction under anchorage-dependent and -independent conditions. Carcinogenesis 1994;15:1001–4.
14. Dong Z, Birrer MJ, Watts RG, et al. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc Natl Acad Sci U S A 1994;91:609–13.
15. Dong Z, Watts SG, Sun Y, et al. Progressive elevation of AP-1 activity during preneoplastic-to neoplastic progression as modeled in mouse JB6 cell variants. Int J Oncol 1995;7:359–64.
16. Dong Z, Lavrovsky V, Colburn NH. Transformation reversion induced in JB6 RT101 cells by AP-1 inhibitors. Carcinogenesis 1995;16:749–56.
17. Dong Z, Cmarik JL. Harvesting cells under anchorage-independent cell transformation conditions for biochemical analyses. Sci STKE 2002;2002:PL7.
18. Lavrovsky V, Dong Z, Ma WY, et al. Drug-induced reversion of progression phenotype is accompanied by reversion of AP-1 phenotype in JB6 cells. In Vitro Cell Dev Biol Anim 1996;32:234–7.
19. Strickland J, Sun Y, Dong Z, et al. Grafting assay distinguishes promotion sensitive from promotion resistant JB6 cells. Carcinogenesis 1997;18:1135–8.
20. Sun Y, Nakamura K, Hegamyer G, et al. No point mutation of Ha-ras or p53 genes expressed in preneoplastic-to-neoplastic progression as modeled in mouse JB6 cell variants. Mol Carcinog 1993;8:49–57.
21. Alblas J, Slager-Davidov R, Steenbergh PH, et al. The role of MAP kinase in TPA-mediated cell cycle arrest of human breast cancer cells. Oncogene 1998;16:131–9.
22. Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–85.
23. Pumiglia KM, Decker SJ. Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A 1997;94:448–52.
24. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807–69.
25. Mousa SS, Mousa SA. Effect of resveratrol on angiogenesis and platelet/fibrin-accelerated tumor growth in the chick chorioallantoic membrane model. Nutr Cancer 2005;52:59–65.
26. Saha P, Banerjee S, Ganguly C, et al. Black tea extract can modulate protein expression of H-ras, c-Myc, p53, and Bcl-2 genes during pulmonary hyperplasia, dysplasia, and carcinoma *in situ*. J Environ Pathol Toxicol Oncol 2005;24:211–24.
27. Mayhew CN, Perkin LM, Zang X, et al. Discrete signaling pathways participate in Rb-dependent responses to chemotherapeutic agents. Oncogene 2004;23:4107–20.
28. Classon M, Harlow E. The retinoblastoma tumor suppressor in development and cancer. Nat Rev Cancer 2002;2:910–7.
29. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell 1995;81:323–30.
30. Hatakeyama M, Weinberg RA. The role of RB in cell cycle control. Prog Cell Cycle Res 1995;1:9–19.
31. Agarwal C, Dhanalakshmi S, Singh RP, et al. Inositol hexaphosphate inhibits growth and induces G_1 arrest and apoptotic death of androgen-dependent human prostate carcinoma LNCaP cells. Neoplasia 2004;6:646–59.
32. Chinni SR, Li Y, Upadhyay S, et al. Indole-3-carbinol (I3C) induced cell growth inhibition, G_1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 2001;20:2927–36.
33. Arakaki N, Toyofuku A, Emoto Y, et al. Induction of G_1 cell cycle arrest in human umbilical vein endothelial cells by flavone's inhibition of the extracellular signal regulated kinase cascade. Biochem Cell Biol 2004;82:583–8.
34. Todd DE, Densham RM, Molton SA, et al. ERK1/2 and p38 cooperate to induce a p21CIP1-dependent G_1 cell cycle arrest. Oncogene 2004;23:3284–95.
35. Rashid MA, Gustafson KB, Cardellina JH, et al. Brominated chamigrane sesquiterpenoids produce a novel profile of differential cytotoxicity in the NCI *in vitro* screen. Nat Prod Lett 1995;6:255–9.
36. Juagdan EJ, Kalidinidi R, Scheuer PJ. Two new chamigranes from an Hawaiian red algae, *Laurencia cartilaginea*. Tetrahedron 1997;53:521–8.
37. Vairappan CS, Suzuki M, Abe T, et al. Halogenated metabolites with antibacterial activity from the Okinawan *Laurencia* species. Phytochemistry 2001;58:517–23.
38. Konig GM, Wright AD. *Laurencia rigida*: chemical investigations of its antifouling dichloromethane extract. J Nat Prod 1997;60:967–70.
39. Konig GM, Wright AD, Franzblau SG. Assessment of antimycobacterial activity of a series of mainly marine derived natural products. Planta Med 2000;66:337–42.
40. Copp BR. Antimycobacterial natural products. Nat Prod Rep 2003;20:535–57.
41. Suzuki M, Daitoh M, Vairappan CS, et al. Novel halogenated metabolites from the Malaysian *Laurencia pannosa*. J Nat Prod 2001;64:597–602.
42. Kimura J, Kamada N, Tsujimoto Y. Fourteen chamigrane derivatives from a red alga, *Laurencia nidifica*. Bull Chem Soc Jpn 1999;72:289–92.
43. Davyt D, Fernandez R, Suescun L, et al. New sesquiterpene derivatives from the red alga *Laurencia scoparia*. Isolation, structure determination, and anthelmintic activity. J Nat Prod 2001;64:1552–5.
44. Lahusen T, De Siervi A, Kunick C, et al. Alsterpaullone, a novel cyclin-dependent kinase inhibitor, induces apoptosis by activation of caspase-9 due to perturbation in mitochondrial membrane potential. Mol Carcinog 2003;36:183–94.

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