Dactylone Inhibits Epidermal Growth Factor–Induced Transformation and Phenotype Expression of Human Cancer Cells and Induces G1-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 G1-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.

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-[5-chamigracen-4-one, IUPAC: (6S,10R)-10-bromo-3,11,11-trimethyl-7-methylidenedienespiro[5,5]undec-2-en-4-one; Fig. 1A] was isolated from the sea hare Aplysia 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 1-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)-2H-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-NH2-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 sample kit was purchased from Cell Signaling Technology, Inc., and the Annexin V-FITC Annexin V-FITC 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% CO2 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% CO2 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.

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 H660 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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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 × 10³/mL) were treated with various concentrations of dactylone in 1 mL of 0.33% basal medium Eagle (BME) agar containing 10% FBS and various concentration of dactylone. The cultures were maintained in a 37°C, 5% CO₂ 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 INCC50 (inhibition of the number of colonies C50) 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 G₁-S phase induced by dactylone was studied by flow cytometry. JB6 P⁺ Cl41 and SK-MEL-28 cells were plated (8 × 10⁵/cm² dish) in 5% (JB6 cells) or 10% 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 FBS. 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 CH1 and human tumor cells was studied using Western blot analysis.

Phosphorylation of ERKs, JNKs, and p38. JB6 Cl41 cells were plated (1 × 10⁶/cm² 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 Na₃VO₄, 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 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), or p38 (Thr180/Tyr182), respectively.

Determination of intracellular levels of cyclin D3, Cdk4, or phosphorylated Rb protein. JB6 C41 cells were plated (1 × 10^4/10-cm dish) in 5% (JB6 C41 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 C41 or SK-MEL-28 cells) or 0.1% FBS/RPMI (H460 cells) for 36 h. Human BSK2-deficient (BSK2−) or wild-type BSK2 cells were plated (1 × 10^4/10-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 C41 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, the 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 (Ser780, Ser795, Ser807/811), 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 C41 cells was evaluated using the luciferase method. Viable JB6 C41 PG-13 (p53), AP-1, or NF-κB cells (6 × 10^5) suspended in 100 μL of 0.1% 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 was 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 pentahydrate (MgCO_3·5H_2O), 5.34 mmol/L MgSO_4·7H_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 FACS Calibur Flow Cytometer (BD Biosciences). JB6 P+ C41 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+ C41) or 10% FBS/MEM (SK-MEL-28 cells). Then, cells were starved in 0.1% FBS/MEM for 12 h (JB6 P+ C41 cells) or 24 h (SK-MEL-28 cells). Cells were then treated with dactylone in 0.1% FBS/medium for 72 h (JB6 P+ C41 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+ C41 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+ C41 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+ C41 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 (INCC50) by dactylone was

Figure 2. Dactylone induces cell cycle arrest at the G1–S transition in JB6 P+ C41 (A) and human skin melanoma SK-MEL-28 cells (B), but not in JB6 C41 DNM-ERK (C) cells. Cell cycle arrest in G1–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 G1, S, or G2 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.
achieved at a concentration of 45.4 μmol/L (Fig. 1A). 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. 1B), colon tumor HCT-116 cells (Fig. 1C), and skin melanoma SK-MEL-5 cells (Fig. 1D) 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. 2A), SK-MEL-28 (Fig. 2B), or JB6 DNM-ERK (Fig. 2C) 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 G1-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. 2A). In contrast, in DNM-ERK cells (Fig. 2C), 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.
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. 3A) at nontoxic concentrations but also in human tumor melanoma (SK-MEL-28) cells (Fig. 3B). 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 G1 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. 4A), p38 (Fig. 4B), and JNKs (Fig. 4C) 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 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 Ser780, Ser807/811, and Ser795 (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.
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. 5D). 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. 6A). 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.

### Discussion

Our data showed that dactylone is representative of a new group of natural cancer-preventive agents. To the best of 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 G1-S cell progression arrest and apoptosis of tumor cells; decreased Rb protein phosphorylation at Ser780, Ser786, and Ser807/811 sites; and inhibited expression of cyclin D3, Cdk4. Active, hypophosphorylated Rb is a potent negative regulator of cell cycle progression from G1 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 Ser786, Ser780, and Ser795, causing G1 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 G1 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 G1-S transition arrest and the lack of inhibition of Rb protein phosphorylation in JB6 Cl41 ERK2 DNMM 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 G1 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 dactyline 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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