

Verrucarin A Inhibition of MAP Kinase Activation in a PMA-stimulated Promyelocytic Leukemia Cell Line

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Received: 4 March 2005 / Accepted: 2 June 2005 / Published: 2 June 2005

Abstract: Verrucarin A is an inhibitor of protein synthesis. In this study, we examined the inhibitory action of verrucarin A on signal molecules. Verrucarin A partially inhibited the IL-8 production of a PMA-stimulated promyelocytic leukemia cell line (HL-60 cells), and the effect was related to the inhibition of NF- κ B activation at non-cytotoxic concentrations. Moreover, the inhibition of mitogen activated protein (MAP) kinase by verrucarin A was especially strong with p38- and JNK-phosphorylation. The findings show a new action of verrucarin A, and it is expected that this action relaxes the signal activation in response to stress.

Keywords: verrucarin A, MAP kinase, HL-60, p38-phosphorylation, JNK-phosphorylation.

Introduction

Marine natural products are important sources of biologically active agents [1]. Microorganisms isolated from marine environments have been recognized as a rich source of biologically active metabolites [2-9]. In the search for new anti-cancer materials, we have screened marine-derived fungal products for natural killer activity. One of about 100 culture broths of marine-derived fungi assayed, strongly inhibited natural killer (NK) activation. Namikoshi et al [10] isolated a new macrocyclic trichothecene, 12,13-deoxyrodin E, and three known compounds (verrucarin A, verrucarins J, and roridin E) from a culture broth of *Myrothecium roridum*. The three known compounds showed strong cytotoxicity in human and murine leukemia cell lines (HL-60 and L1210, respectively), but 12,13-deoxyroridin E was about 80-fold less cytotoxic than roridin E, the epoxide variant. Many natural bioactive compounds are toxic to mammalian cells but at specific concentrations are expected to have a specific activity.

In this study, we demonstrated that verrucarins A inhibited the activation of p38 and JNK in the cytosol fraction. From this result we anticipate the discovery of a new compound that confers resistance to the excessive stress reaction in mammalian cells.

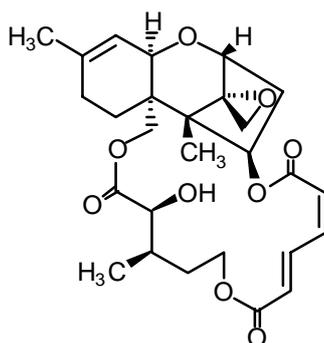


Figure 1. Structure of verrucarins A.

Materials and Methods

Material

Verrucarins A, verrucarins J and roridin E were prepared as reported [10]. The structure of verrucarins A is shown in Figure 1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 12-O-tetradecanoyl phorbol 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Sodium [⁵¹Cr]chromate was obtained from Amersham Biosciences K.K. (Tokyo, Japan) Antibodies to IκB-α, phospho-p38, phospho-JNK, phospho-ERK1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents and chemicals used were of the highest grade available commercially.

Cell lines and culture conditions

The human promyelocytic cell line HL-60 and human erythroleukemia cell line K562 were obtained from the Japanese Cancer Research Resources Bank (JCRB, Kamiyoga, Tokyo, Japan). These cell lines were maintained in tissue culture dishes in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml of penicillin G and 100 µg/ml of streptomycin.

NK assay

The NK-susceptible target K562 tumor cells were labeled with ⁵¹Cr. Peripheral blood leukocytes (PBLs) were prepared from human blood. The PBLs (2 x 10⁵ cells/100 µl medium) were plated, and IL-2 (to a final concentration of 10 ng/ml) was added. Then, the test compound was dissolved in DMSO and added to the PBL suspension (1 µl) at a final volume of 1 µl, and the cells were cultured overnight. ⁵¹Cr-labeled K562 cells were added to each well and incubated at 37°C for 4.5 hr. The plate was centrifuged, and 100 µl of supernatant was used for the analyses in a gamma counter [11].

The cytotoxicity was calculated as follows.

$$\% \text{ Cytotoxicity} = \frac{\text{Release in experiment} - \text{Release in medium alone}}{\text{Maximum release} - \text{Release in medium alone}} \times 100$$

Detection of human IL-8 by ELISA

The interleukin 8 (IL-8) concentration of the culture supernatants under control and various test conditions was measured by ELISA using a combination of monoclonal and biotinylated monoclonal antibodies [12]. All samples were assayed at least in duplicate. Data are presented as the mean ± SE of three independent experiments.

Determination of cytotoxicity

Lethal cell injury was assessed by measuring lactate dehydrogenase (LDH) release [13], while proliferation was evaluated by enumerating viable cells using the MTT formazan production method [14]. HL-60 cells (1 x 10⁶ cells/ml) were treated with PMA (with or without verrucarin A) and then transferred to 96-well microtiter plates. After a 24 h incubation, the LDH activity in the supernatants was determined, and 20 µl of MTT reagent (5 mg/ml in PBS) was then added to each well. After incubation for 3 h, formazan production was assessed by measuring the optical density (OD₅₇₀ nm).

Western blot analysis

HL-60 cells were treated with verrucarin A and lysed in a buffer. The amount of protein in each sample was determined using the Bradford assay. Equal amounts of lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto nitrocellulose filters. After blocking with 5% non-fat milk in tris-buffered saline at room temperature for 1 h, the membranes were probed with antibodies to pp38, pJNK, and pERK1. They were then incubated for 30 min with anti-rabbit Ig (Amersham Biosciences K.K., Tokyo, Japan) and for another 30 min with peroxidase-streptavidin conjugate. Blots were developed using an enhanced chemiluminescence detection system (Amersham Biosciences K.K., Tokyo, Japan).

Plasmid construction, cell transfection and luciferase assay

Plasmid construction, cell transfection and the luciferase assay were carried out as described previously [15]. Luciferase reporter plasmids containing the luciferase gene under the control of promoters containing the recognition sites for nuclear factor (NF)- κ B were constructed. Oligonucleotides corresponding to the recognition site for each of these factors were synthesized using a DNA synthesizer (RIKAKEN CO., LTD., Tokyo, Japan). The sequences were: 5'-AAGGGACTTTCCGCTGGGGATTCCAG-3' (NF- κ B, four copies). This resulted in a reporter plasmid in which the expression of the luciferase gene was under the control of the relevant transcription factors.

For cell transfections and luciferase assays, a modification of a previously described method [16] was used. Transfection was performed by the electroporation method. HL-60 cells (2.5×10^7) were washed with RPMI 1640 medium and resuspended in 700 μ l of the medium containing 20 μ g of reporter plasmid DNA. Electroporation was performed at 960 mF and 340 V with a Gene Pulser and a Gene Pulser Cuvette with a 0.4 cm electrode gap (Bio-Rad, Hercules, CA, USA). The cells were suspended in 40 ml of RPMI 1640 medium containing 10% FBS, divided between six 6-cm dishes and incubated for 24 h before being treated with the stimulants and/or test compounds. Cell extracts were prepared 8 h after treatment with PMA, and their luciferase activity was measured using a Pica Gene detection kit (Tokyo Inc. Tokyo, Japan) and a MiniLumat LB 9506 (Berthold Japan K.K., Tokyo, Japan).

Results

The effects of marine-derived fungal products on human NK activity

In order to identify new anti-cancer compounds, an NK assay that uses the T cell fraction of NK cells isolated from human blood, was performed with about 100 culture broths of marine-derived fungi. K562 as a target cell was labeled with ^{51}Cr , and [^{51}Cr] K562 and T cells (contained in NK cells) were mixed at a fixed ratio in the presence of IL-2; the radioactivity in the supernatant correlated with NK activity. As the control, we determined the radioactivity when DMSO was added to a mixture of

T and [^{51}Cr]K562 cells. The other samples were used for the change of DMSO. The results for about 100 samples are summarized in Table 1. They showed that no fungal broth strengthened NK activation, as expected, but that strong inhibition was shown by some broths. Notably, No. 42 was of interest, because no inhibition was obtained when IL-2 was not added, but a strong inhibition was detected when IL-2 was present (Table 1). Many natural bioactive compounds are toxic to mammalian cells, but specific concentrations are expected to have a specific action.

Table 1. Effect of filamentous fungal broths on NK activity by human peripheral blood lymphocytes.^a

Broth No.	Relative activity (%)	
	IL-2 added	IL-2 not added
Control	100	100
9	7	0
42	0	154
57	32	58
61	34	38
74	1	17
75	4	0
93	43	0
94	24	24

^a The experimental conditions for determination of NK activity were described in the experimental section. The data are means of two independent experiments.

The effects of roridin E, verrucarin A and verrucarin J on IL-8 production by PMA-stimulated HL-60 cells

Namikoshi et al. isolated a new macrocyclic trichothecene, 12,13-deoxyroridin E, and three known compounds (verrucarin A, verrucarin J, and roridin E) from a culture broth (No.42) of *Myrothecium roridum* (2002). The three known compounds showed strong cytotoxicity in human and murine leukemia cell lines (HL-60 and L1210, respectively), but 12,13-deoxyroridin E was about 8 fold less cytotoxic than roridin E, the epoxide variant.

Therefore, the effects of verrucarin A, verrucarin J and roridin E on IL-8 production by PMA-stimulated HL-60 cells were examined, and the results are shown in Figure 2A, C and E. Verrucarin A and roridin E inhibited IL-8 production by PMA-stimulated HL-60 cells, but the inhibition by roridin E and verrucarin A (0.1 and 1 $\mu\text{g}/\text{ml}$) correlated with cell proliferation in the MTT assay. Therefore, it is estimated that the inhibition by roridin E and verrucarin A (0.1 and 1 $\mu\text{g}/\text{ml}$) is due to their cytotoxicity. On the other hand, verrucarin A (1 and 10 ng/ml) did not affect cell proliferation. Therefore, verrucarin A was of interest.

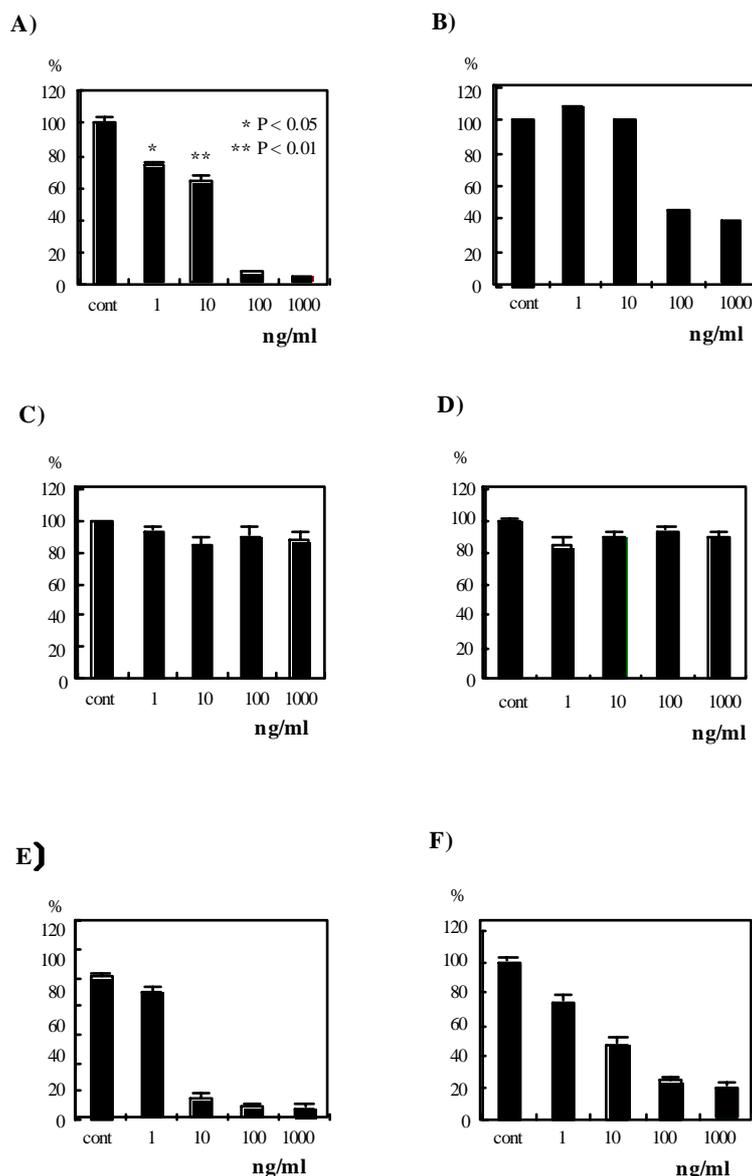


Figure 2. Effects of verrucarins A, verrucarins J and roridin E on IL-8 production and cell proliferation in PMA-stimulated HL-60 cells. A), C) and E) show effects of verrucarins A, verrucarins J and roridin E, respectively, on IL-8 production. HL-60 cells (1×10^6 cells/ml) were treated with PMA (20 nM) and the indicated concentrations of the compounds for 24 h. The IL-8 concentration in the culture supernatant of the PMA-stimulated cells was determined to be ca. 24 ng/ml by ELISA as described in Materials and Methods. The data are shown as values relative (%) to the PMA-stimulated IL-8 production (24 ng/ml). The data are the mean values of three independent experiments. B), D) and F) show the effects of verrucarins A, verrucarins J and roridin E, respectively, on cell proliferation. The MTT assay was as described in Materials and Methods. The data are shown as values relative (%) to the value of PMA-stimulated condition. The data are the mean values of three independent experiments.

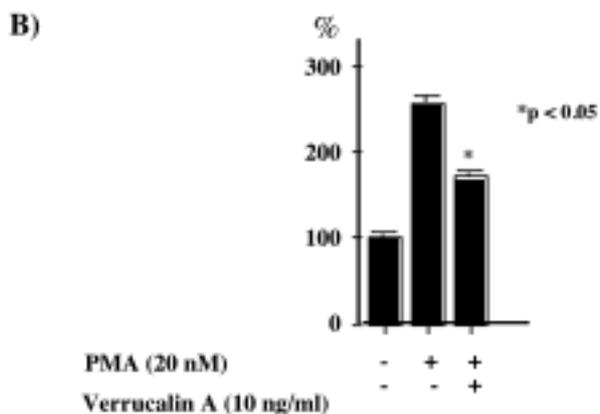
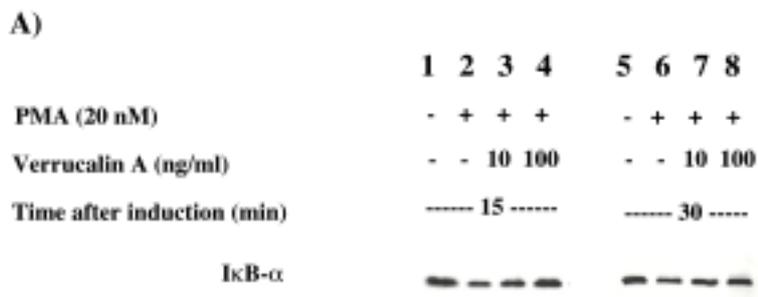


Figure 3. Effect of verrucarin A on the activation of NF-κB in PMA-stimulated HL-60 cells.

A) Western blot analysis with polyclonal antiserum to IκB-α. Total protein extracted from the control (1 and 5) and PMA-stimulated HL-60 cells, with or without 15 or 30 min incubation with verrucarin A (2-4 and 6-8), was electrophoresed on 10% SDS-PAGE gels and examined by western blotting using polyclonal antiserum. B) Effect of verrucarin A on the transcriptional activation of the NF-κB binding region in PMA-stimulated HL-60 cells. HL-60 cells (2.5×10^7) were transfected with the reporter plasmid including the NF-κB binding region and treated with PMA (20 nM) and verrucarin A. The luciferase activities were determined as described in Materials and Methods. The activity of the cells treated with PMA (20 nM) was set at 100%. Each value is the average of the results from three independent experiments.

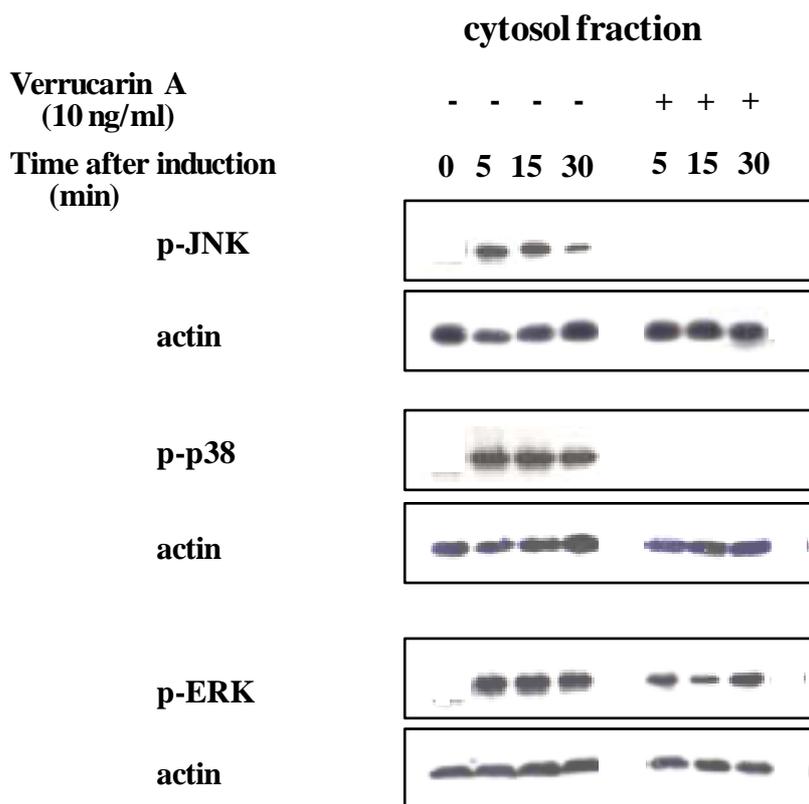


Figure 4. Effect of verrucarín A on the activation of the MAP kinase in PMA-stimulated HL-60 cells as determined by western blotting using phosphorylated anti-p38, -JNK and -ERK antibodies.

Protein in the cytosol protein preparations treated with or without verrucarín A was analyzed by western blotting.

Inhibitory mechanism of verrucarín A for PMA-stimulated IL-8 production

Recently, it was revealed that NF- κ B and AP-1 are important for the production of IL-8 [17]. We first investigated whether verrucarín A affects the activation of NF- κ B. Inactive NF- κ B complexes are associated with inhibitory proteins of the I κ B family, which sequester NF- κ B in the cytoplasm. It is well known that in response to various stimuli, I κ B proteins are first phosphorylated, then ubiquitinated and finally, rapidly degraded by the proteasome, allowing for the nuclear translocation of NF- κ B and transcriptional initiation of NF- κ B-dependent genes. The I κ B family includes the widely distributed I κ B- α and I κ B- β proteins, together with I κ B- γ , I κ B- ϵ and Bcl-3. I κ B proteins are characterized by a series of ankyrin repeats that interact with the DNA binding domain and nuclear localization signal of NF- κ B. Therefore, the effect of verrucarín A on the activation of NF- κ B was determined by western blotting with anti I κ B- α . The results, shown in Figure 3A, indicate that verrucarín A slightly inhibited the degradation of I κ B- α . We also investigated the effect of verrucarín A on the transcriptional activity of the IL-8 promoter region using NF- κ B binding of the promoter

region. The results are shown in Figure 3B. The findings indicated that verrucarin A partially inhibited IL-8 production at the transcriptional level in PMA-stimulated HL-60 cells. Next, the effects of verrucarin A on the activation of MAP kinase were examined. We prepared cytosol fractions for each experimental conditions, and analyzed them by Western blotting with antibodies for phosphorylated p38, JNK and ERK. The results are shown in Figure 4. In the cytosol fraction, the activation of JNK and p38 was inhibited more than the activation of ERK-1.

Discussion

We identified verrucarin A by a natural killer (NK) cell assay in marine-derived fungal products. Recently, verrucarin A was reported to be an inhibitor of protein synthesis, and to affect inflammatory cytokine production [18,19], but its target molecule for the inhibition of inflammation was not clear. Therefore, we examined the effects of verrucarin A on IL-8 production in PMA-stimulated HL-60 cells. The results indicated a weak inhibition of IL-8 production at concentrations that were not cytotoxic. Therefore, the target for inhibition was JNK and p38 MAP kinases. It was supposed that the fraction No.42 (increased verrucarin A) inhibited IL-2 dependent MAP kinase activation in NK cells. Although, p42 MAP kinase inhibitor (PD 09805) and p38 MAP kinase inhibitor (SB 203580) have recently been reported, verrucarin A inhibited both p38 and JNK MAP kinases. Generally, the activation of JNK and p38 MAP kinase is promoted by stress, and thus verrucarin A is expected to inhibit the activation of MAP kinase (JNK and p38).

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Sample availability: Not available.