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Ammocidin, a New Apoptosis Inducer in Ras-dependent Cells from *Saccharothrix* sp.

I. Production, Isolation and Biological Activity

RYO MURAKAMI, TAIJIRO TOMIKAWA, KAZUO SHIN-YA, JUNKO SHINOZAKI†, TAKAYUKI KAJIURA†, TAISEI KINOSHITA, ATSUSHI MIYAJIMA, HARUO SETO and YOICHI HAYAKAWA*

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan
† Pharmaceutical Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-shi, Kanagawa 210-8681, Japan

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A new apoptosis inducer, ammocidin, was isolated from the culture broth of *Saccharothrix* sp. AJ9571. Ammocidin induced apoptotic cell death in Ras-dependent Ba/F3-V12 cells with an IC_{50} of 66 ng/ml. No cell death was observed in IL-3-dependent Ba/F3-V12 cells at less than 100 μg/ml of ammocidin. Ammocidin significantly reduced the phosphorylation level of MAPK and S6K that mediate the anti-apoptotic function of Ras.

Certain mutations in the *ras* proto-oncogene are frequently encountered in human cancer cells, especially in colon or pancreas cancer. Activated Ras is able to transform most immortalized cell lines and plays a key role in mitogenic signal-transduction pathways[1]. The oncogenic Ras also functions as an apoptosis suppressor in certain conditions[2~4]. Thus, selective apoptosis inducers in Ras-dependent cells may be useful as anticancer agents against cells expressing the mutant Ras. Hematopoietic cell-lines are appropriate for constructing a model of Ras-dependent cells, because viability and proliferation of hematopoietic cells are strictly dependent on cytokines such as interleukins (IL) and colony-stimulating factors (CSF). The IL-3-dependent Ba/F3 pro-B cells undergo rapid apoptosis in the absence of IL-3. However, Ba/F3 cells bearing dexamethasone-inducible v-H-Ras (Ba/F3-V12) completely survive in an IL-3-free medium containing dexamethasone (Dex)[2]. In the course of our screening for apoptosis inducers in Ras-dependent Ba/F3-V12 cells, a new active substance designated ammocidin (Fig. 1) was isolated from the culture broth of *Saccharothrix* sp. AJ9571. This paper describes the production, isolation and biological activity of ammocidin. The physico-chemical properties and structure elucidation of ammocidin are described in the accompanying paper[5].

Materials and Methods

Microorganism

The producing organism designated AJ9571 was isolated from a soil sample collected at Tottori Sand Hill, Japan. Strain AJ9571 was identified as *Saccharothrix* sp. on the basis of culturål, physiological and chemical characterization[6~10].

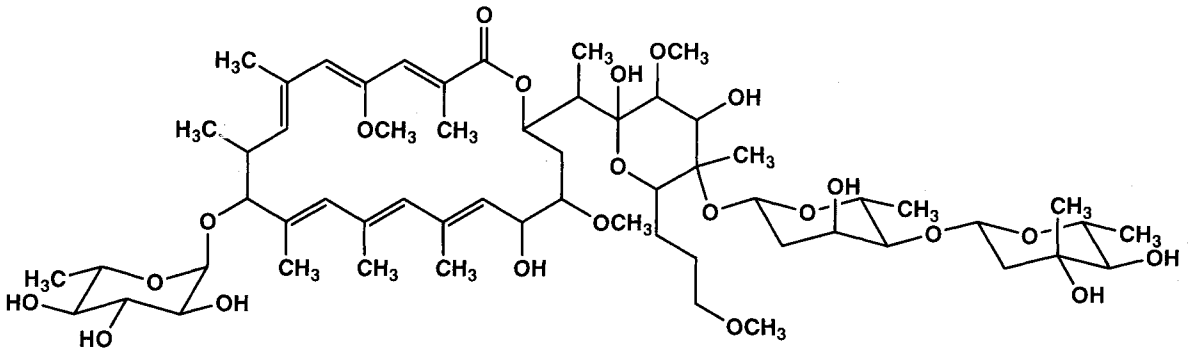
Cell Culture and Bioassay

Ba/F3-V12 cells were cultured in RPMI-1640 medium supplemented with heat-inactivated fetal calf serum (8%), mouse IL-3 (2 ng/ml), 2-mercaptoethanol (50 μM), G418 (0.1 mg/ml), penicillin G (1000 units/ml) and streptomycin (0.1 mg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

The cells at 2.5×10^4 cells/ml were put into each well of 96-well plates. After incubation with various concentrations of samples at 37°C for 48 hours, the cells were treated with 0.5 mM of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt

* Corresponding author: hayakawa@iam.u-tokyo.ac.jp

Fig. 1. Structure of ammocidin.



(WST-8) at 37°C for 1 hour. The relative cell number was measured with formazan formation at 450 nm using a multilabel counter (Wallac 1420 ARVOsx, Perkin Elmer, Inc.).

Apoptosis Assays

Ba/F3-V12 cells (2×10^6 cells) were fixed in 100 μl of 1% glutaraldehyde-PBS at room temperature for 30 minutes. The cells were precipitated by centrifugation and suspended in 20 μl of PBS. Chromatin structure was visualized by fluorescence microscopy (Axiovert 135, Carl Zeiss Co. Ltd.) after staining the cells with 4 μl of Hoechst Dye 33258 (1 mM).

Ba/F3-V12 cells (5×10^6 cells) were lysed with 600 μl of a buffer (pH 7.5) consisting of 10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100. Insoluble materials were removed by centrifugation and the supernatant was twice extracted with phenol-chloroform (1:1) followed by ethanol precipitation. The pellet was dissolved in 20 μl of a buffer (pH 8.0) consisting of 10 mM Tris-HCl, 1 mM EDTA and 2 μg/ml RNaseA, and incubated at 37°C for 30 minutes. The samples were loaded onto a 2.0% agarose gel for electrophoresis and fragmented DNA was visualized by ethidium bromide staining.

Western Blotting

Expression of Ras and phosphorylation of MAPK and S6K were assessed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ba/F3-V12 cells were deprived of IL-3 for 2 hours and then stimulated with 2×10^{-7} M Dex. The cells were lysed with 100 μl of a lysis buffer (pH 7.4) consisting of 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 30 mM Na-pyrophosphate, 50 mM NaF, 1 mM Na-orthovanadate, 0.1 mM Pefabloc and 5 μg/ml leupeptin. After 45 minutes of gentle agitation at 4°C, insoluble materials were cleared by centrifugation. An aliquot of each sample was loaded onto an SDS-PAGE system. Protein levels for Ras, MAPK, S6K, phosphorylated MAPK and phosphorylated S6K were detected by Western blotting using specific antibodies against H-Ras, ERK2, p70/S6K (Santa Cruz Biotechnology), pT^{183} MAPK (Promega) and phospho-p70 S6 kinase (Thr389) (Cell Signaling Technology).

Results and Discussion

Production

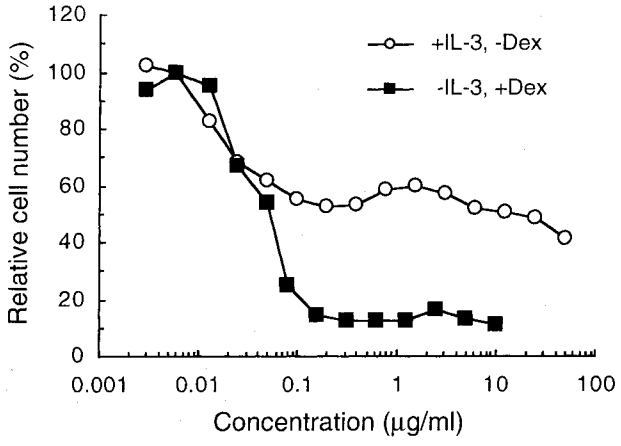
A seed medium was composed of yeast extract 0.1%, beef extract 0.1%, N-Z amine Type A 0.2% and glucose 1.0% (pH 7.3). *Saccharothrix* sp. AJ9571 was cultured in flasks containing the seed medium on a rotary shaker at 30°C for 4 days. The resultant seed culture at 2% was transferred into 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of glycerol 2.0%, starch 1.0%, rape seed meal 2.0%, Soytone 0.3% and calcium carbonate 0.3% (pH 7.0). The fermentation was carried out on a rotary shaker at 30°C for 7 days.

Isolation

The culture broth (10 liters) was centrifuged and the supernatant was extracted with ethyl acetate. The mycelial acetone extract was concentrated to a small volume and then extracted with ethyl acetate. The combined solvent extract was subjected to silica gel column chromatography

Fig. 2. Effect of ammocidin on the viability and growth of Ba/F3-V12 cells.

Ba/F3-V12 cells were cultured for 48 hours with various concentrations of ammocidin and then the relative cell number was measured by the WST-8 assay.



with chloroform-methanol (8:1). The active eluate was chromatographed on a Sephadex LH-20 column with MeOH. The active fraction was purified by HPLC using a Shenshu Pak PEGASIL ODS column (20 i.d.×250 mm) with 70% MeOH. Further purification was carried out on the same column with 33% acetonitrile to give a colorless powder of ammocidin (387 mg).

Biological Activity

Ammocidin induced cell death in Ba/F3-V12 cells in an IL-3-free medium containing Dex (2×10^{-7} M) with an IC_{50} of 66 ng/ml. No cell death was observed in the presence of IL-3 and less than 100 μg/ml of ammocidin, although ammocidin inhibited the growth of IL-3-dependent Ba/F3-V12 cells (Fig. 2). During apoptosis, loss of membrane integrity is typically preceded by chromatin condensation and internucleosomal cleavage of genomic DNA[11]. Significant numbers of Ras-dependent Ba/F3-V12 cells treated with 1 μg/ml of ammocidin for 20 hours contained condensed chromatin and fragmented nuclei as visualized by staining with Hoechst Dye 33258 (Fig. 3). The extract of these cells contained a large amount of fragmented DNA (Fig. 4). These data indicate that cell death induced by ammocidin resulted from apoptosis.

The anti-apoptotic function of Ras in Ba/F3 cells is mediated by mitogen-activated protein kinase (MAPK) and S6 kinase (S6K) pathways[12]. The Ras expression by Dex

Fig. 3. Fluorescence micrographs of Ba/F3-V12 cells stained with Hoechst Dye 33258.

Ba/F3-V12 cells were cultured with (bottom) or without (top) 1 μg/ml of ammocidin for 20 hours after IL-3 withdrawal and Dex addition.

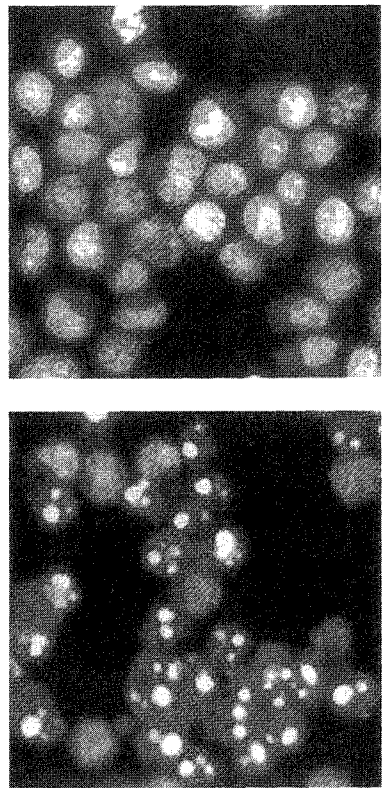


Fig. 4. Ethidium bromide-stained DNA extracted from Ba/F3-V12 cells.

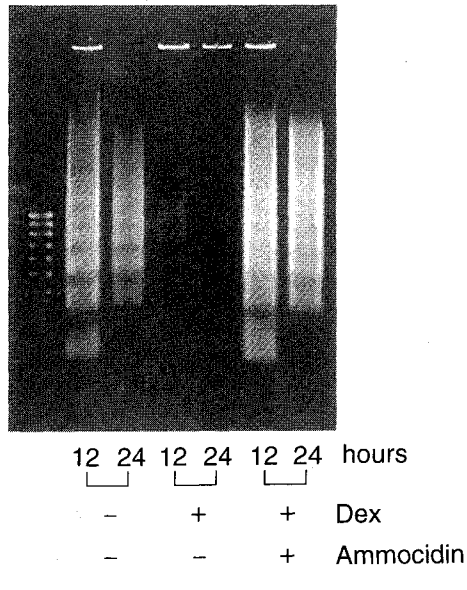
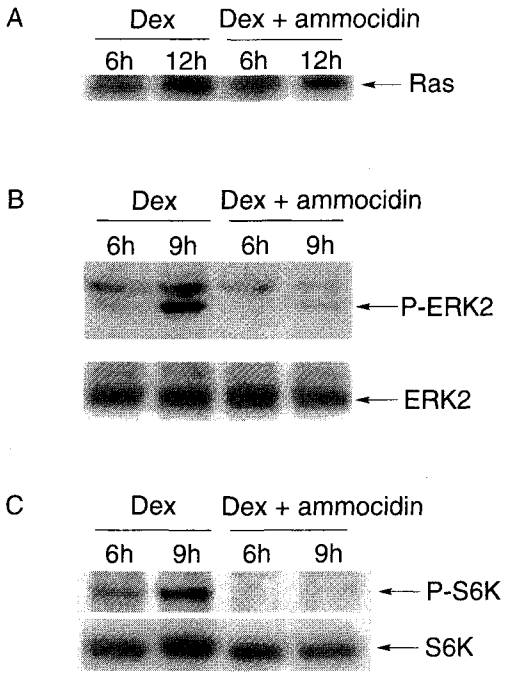


Fig. 5. Effect of ammocidin on Ras expression (A) and phosphorylation of ERK2 (B) and S6K (C).



clearly induced phosphorylation of MAPK and S6K. However, the phosphorylation level of MAPK and S6K was significantly reduced in the presence of 1 μg/ml of ammocidin (Fig. 5). These results suggest that ammocidin-induced apoptosis in Ras-dependent cells is due to simultaneous inhibition of MAPK and S6K phosphorylation by ammocidin. Further studies on the biological activity of ammocidin are in progress.

Acknowledgements

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