J. Microbiol. Biotechnol. (1998), 8(5), 455-460

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY
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Aburatubolactam C, a Novel Apoptosis-inducing Substance Produced by Marine *Streptomyces* sp. SCRC A-20

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Received: April 21, 1998

Abstract In the course of screening for new antitumor substances, a novel cytotoxic agent inducing apoptotic cell death was isolated from the culture broth of marine bacterial strain SCRC A-20. Strain SCRC A-20 was separated from a mollusk and was chemotaxonomically identified as a *Streptomyces* sp. The cytotoxic substance was purified by organic solvent extraction followed by silica gel column chromatography and preparative TLC. HRFAB-MS determined its molecular formula to be $C_{30}H_{40}N_2O_5$ (MW 508). The 1D and 2D NMR spectral data demonstrated that the substance has a novel lactam structure of a 20-membered macrocycle coupled with a unique acyl tetramine and bicyclo[3.3.0] octane, which includes three methyl groups, six olefinic protons, five carbonyl groups, a conjugated diene and a dienone. The substance, named aburatubolactam C, appeared to be cytotoxic for various continuously proliferating tumor cells of human and murine origins. The IC_{50} values determined by MTT assay were in the range of 0.3 to 5.8 μg/ml. When Jurkat T cells were treated with 3 μg/ml of aburatubolactam C, the apoptotic DNA fragmentation was detectable within 3 h, indicating that the cytotoxic effect of aburatubolactam C on tumor cells is attributable to the induced apoptosis.

Key words: Aburatubolactam C, marine bacterium, cytotoxicity, apoptosis, antitumor agent

Apoptosis is a form of programmed cell death that is accompanied with a distinctive series of morphological changes involving cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation with internucleosomal DNA digestion [1, 22]. Since chemotherapy for tumors is principally based on agents that are toxic to the cells and since induction of apoptosis in tumor cells can lead to their own destruction, apoptosis has been recently implicated as an important cellular target for antineoplastic drugs. It has been shown that several anticancer drugs such as etoposide, VM26, m-AMSA, dexamethasone, vincristine, cis-platium, cyclophosphamide, paclitaxel, 5'-fluoro-deoxyuridine, 5'-fluorouracil, and adriamycin induce extensive apoptosis rapidly in tumor cells [5, 7, 8, 10, 11, 19, 21].

A majority of these known anticancer drugs are among natural cytotoxic substances isolated from soil microorganisms and plants, which were previously main targets for the screening of structurally unique and biologically active novel compounds including antitumor agents. Recently, several leading countries have started to employ marine resources in isolating novel bioactive compounds [4, 16, 17]. Despite a relatively short history, the structures of over 5500 novel substances have been determined from marine resources. However, since less than 1% of those novel substances appear to originate from marine microorganisms and only a small number of the substances are known to have anticancer activity, it is likely that marine microorganisms could be a new target for the screening of novel substances including anticancer drugs.

We also employed microorganisms from marine resources to find a novel natural compound that has cytotoxic effect on tumor cells through inducing apoptosis. During the screening for the cytotoxic substance from marine microorganisms, we succeeded in isolating a novel cytotoxic agent, alteramide, which is produced by *Alteromonas* sp. SCRC K280 and possesses cytotoxicity against several tumor cells [18]. From marine bacterial strain *Streptomyces* sp. SCRC-A20 we isolated a novel substance, aburatubolactam A, which has a unique lactam

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structure and an inhibitory effect on superoxide anionic generation in human neutrophils [2]. In addition, our continuous search for structurally unique substances from the strain SCRC A-20 enabled us to identify aburatubolactam C which also has a lactam structure similar to that of aburatubolactam A.

Recently, we have shown that aburatubolactam C has cytotoxicity against several human and murine tumor cell lines, and rapidly induces apoptosis in Jurkat T cells through upregulation of Fas ligand expression and subsequent activation of Fas death signal [3]. In the present study, we describe the taxonomy of the marine bacterial strain producing aburatubolactam C, a purification procedure, the physicochemical properties and the biological activities of the substance.

MATERIALS AND METHOD

Microorganism and Taxonomy

Marine bacterial strain SCRC A-20 producing cytotoxic agent aburatubolactam C was isolated from a mollusk sample collected in Aburatubo Bay, Japan. The taxonomic studies were carried out as described by the International *Streptomyces* Project [15, 20]. The physiological characteristics including utilization of carbon sources were examined by the method of Pridham and Gottlieb [14]. Analysis of cell wall components and cellular membranous fatty acids was performed by the methods of Goodfellow *et al.* [12].

Bacterial Media and Culture Conditions

A loopful of strain SCRC A-20 grown on an agar slant was inoculated into five 500-ml Erlenmeyer flasks containing 80 ml of sterile seed medium consisting of 1.0% glucose, 0.5% bactopeptone, 0.1% yeast extract, and 75% artificial sea water (adjusted to pH 7.4 before sterilization) and incubated on a rotary shaker at 25°C for 2 days at 150 rpm. For the production of aburatubolactam C, 20 ml of seed culture was transferred to twenty 3-liter Erlenmeyer flasks containing 500 ml of the same medium, and incubated for 7 days under the same conditions.

Instrumental Analysis

IR spectra were measured with a Jasco IR-80 (JASCO, Japan) spectrometer. Mass spectra were taken on a Hitachi M-80A (Hitachi, Japan) and JMS-DX 303HF (JEOL, Japan) spectrometer. Optical rotation was taken with a Horiba SEPA-200 polarimeter with a 5-cm micro cell. ^{1}H-NMR, ^{1}H-^{13}C-COSY, ^{13}C-NMR and HMBC spectra were recorded with a GSX-400 (JEOL, Japan) spectrometer, using pyridine-d_5 and chloroform-d as solvents. Silica gel (Merck Kieselgel 60, 70-230 mesh) and silica TLC plates (Silica gel 60 F_{254}) were purchased from Merck Company (Darmstadt, Germany).

Mammalian Cell Culture

Human Jurkat T cells, human myeloid leukemia U937 cells, and murine lymphoma BW5.1.4.7.G.1.4 cells were kindly supplied by Dr. Albert A. Nordin (Gerontology Research Center, NIA/NIH, Baltimore, MD, U.S.A.). Human promyelocytic leukemia HL-60 cells, human chronic myelogenous leukemia K562 cells, colon adenocarcinoma COLO 320DM cells, human hepatocellular carcinoma HepG2 cells, murine lymphoid neoplasm P388D1 and murine fibroblast NIH3T3 cells were from Korean Collection for Type Culture (KCTC, KRIBB, Taejon, Korea). Jurkat, U937, HL-60 and K562 cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD, U.S.A.) containing 10% FBS (UBI, Lake Placid, NY, U.S.A.), 20 mM HEPES (pH 7.0), 5×10^{-5} M β-mercaptoethanol, and 100 μg/ml gentamycin. The culture medium used for BW5.1.4.7.G.1.4, P388D1 and NIH3T3 cells was Dulbecco's Modified Eagles Medium (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 10% FBS, 20 mM HEPES (pH 7.0), 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, and 100 μg/ml gentamycin. HepG2 cells were maintained in MEM (Life Technologies, Gaithersburg, MD, U.S.A.) containing 10% FBS, 20 mM HEPES (pH 7.0), 1 mM nonessential amino acid solution (Life Technologies, Gaithersburg, MD, U.S.A.), 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, and 100 μg/ml gentamycin.

Cytotoxicity Assay and DNA Fragmentation Analysis

The cytotoxic effect of aburatubolactam C on several tumor cells was analyzed by MTT assay [13], which is a method to determine the viability of cells by measuring the capacity of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the colored formazan crystal. Tumor cells were grown for 2 days with or without the compound to be tested. After incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. After centrifugation, the supernatant was removed from each well and then 150 μl of DMSO was added to dissolve the colored formazan crystal produced from MTT. OD values of the solutions were measured at 540 nm by a plate reader.

To determine apoptotic DNA fragmentation induced in Jurkat T cells following the treatment by aburatubolactam C, the isolation of apoptotic DNA fragments was performed as described by Herrmann *et al.* [6]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min at $1600\times g$, the supernatant was collected and brought to

1% SDS, and treated for 2 h at 50°C with RNase A and subsequently with proteinase K for 2 h at 37°C. The DNA fragments were precipitated with 2.5 volume of ethanol in the presence of 5 M ammonium acetate. The DNA fragmentation was visualized by electrophoresis on a 2% agarose gel as described elsewhere [6].

Results and Discussion

Taxonomy of the Producing Strain

The taxonomical characteristics of marine bacterial strain SCRC A-20 are summarized in Table 1. The colony morphology of strain SCRC A-20 grown on yeast malt agar at 25°C for 3 days was fawn/pale brown, opaque, round, regular, entire, rampartlike, wrinkled, and matt. Strain SCRC A-20 was Gram-positive and immobile. The colonies were 1.0~1.5 mm in diameter. The growth temperature range was 10~45°C. The strain was positive for the decomposition of starch and skim milk, but negative for gas production from glucose. The strain utilized D-glucose, D-xylose, L-arabinose, cellobiose and cellulose, but did not utilize D-fructose and inositol. Although these results suggest that strain SCRC A-20 may belong to either *Norcardiodes* or *Streptomyces*, the morphological property showing the easy breaking-up of mycelium into fragments indicates that SCRC A-20 could belong to the genus *Nocardiodes*.

To further confirm the assignment of strain SCRC A-20 to *Nocardiodes*, its chemotaxonomical characteristics were investigated and compared with those of *Nocardiodes letus*. Strain SCRC A-20 appeared to contain no mycolic acids, but possessed an LL-diaminopimelic acid isoform in the cell wall. As shown in Fig. 1A, cellular fatty acids of strain SCRC A-20 were composed of three major fatty acids, 12-methyltetradecanoic (anteiso-C15:0), 14-metylpentadecanoic (iso-C16:0) and 14-metylhexadecanoic (anteiso-C17:0) acids. The fatty acid profile of SCRC A-20 was significantly different from that of *N. letus* because *N. letus* did not have significant amounts of 12-methyltetradecanoic acid (anteiso C15:0) but contained significant amounts of octdecenoic acid (C18:1) (Fig. 1B). However, the fatty acid composition of SCRC A-20 appeared to be similar to that of *Streptomyces* which has been described previously [9, 12]. These results suggest that SCRC A-20 is chemotaxonomically closer to *Streptomyces* than to *Nocardiodes*, although a morphological property regarding mycelium fragmentation does not rule out the possibility that SCRC A-20 may belong to *Nocardiodes*. Thus, based on chemotaxonomical data, we identified strain SCRC A-20 as a new species of genus *Streptomyces*.

Table 1. Taxonomical characteristics of strain SCRC A-20.

Conditions	Characteristics
Gram stain	Positive
Sporulation	Negative
Motility	Negative
Aerial mycelium	Present
Hydrolysis of skim milk	Positive
Gas production from glucose	Negative
Temperature range for growth	10~45°C
DAP type	LL
Garbon utilization	
D-Glucose	+
D-Fructose	–
Cellulose	+
Inositol	–
D-Xylose	+
L-Arabinose	+
Cellobiose	+

+, positive utilization; –, no utilization.

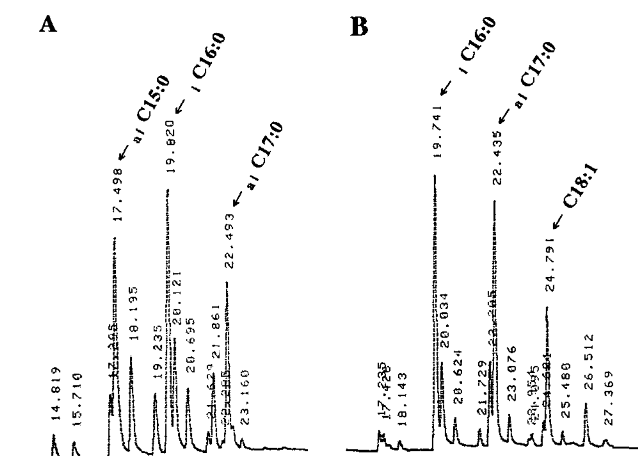

Fig. 1. Gas-liquid chromatogram of cellular fatty acids of marine bacterial strain SCRC A-20 (A) and *Nocardiodes letus* (B).

Isolation and Purification

The procedure used for the isolation of aburatubolactam C is shown in Fig. 2. The cultured broth (10 liter) of strain SCRC A-20 was centrifuged at 8000 rpm to remove mycelium. The supernatant was extracted with the same volume of ethyl acetate three times. The extracts were combined and concentrated under reduced pressure, and approximately 1.9 g of powder was recovered. The ethyl acetate extract was dissolved in 10 ml of chloroform and methanol solution (96:4, v/v), and then fractionated by column chromatography on silica gel, which was eluted stepwise with three different mixtures of chloroform and methanol such as 96:4, 92:8, and 80:20, v/v. The active fraction eluted with 600 ml of chloroform and methanol solution (92:8, v/v) was concentrated under reduced pressure and 84 mg of powder was obtained. The powder was dissolved in a small amount of chloroform and then applied to a

preparative TLC (developing solvent, $CHCl_3$:Et_2NH=7:3 v/v). The band with the Rf value of 0.45 was scraped and was extracted with chloroform:methanol solution (2:1, v/v). After the extract was concentrated under reduced pressure, 14 mg of the active cytotoxic agent was obtained as a white solid.

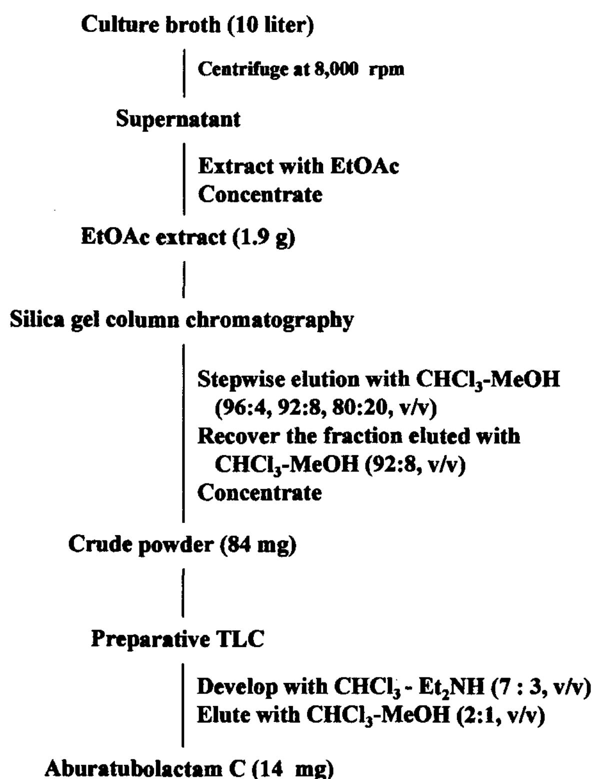

Fig. 2. Isolation procedure of aburatubolactam C from the culture broth of *Streptomyces* sp. SCRC A-20.

Physicochemical Properties and Structure Elucidation

Physicochemical properties of the active cytotoxic substance are summarized in Table 2. The cytotoxic substance was soluble in chloroform, ethyl acetate and DMSO, and slightly soluble in methanol and ethanol. However the substance was insoluble in water and *n*-hexane. The MW and formula of the cytotoxic substance was determined to be $C_{30}H_{40}N_2O_5$ (MW 508) by HRFAB-MS. The IR spectra demonstrated the existence of hydroxyl (3465 cm^{-1}), ester (1735 cm^{-1}) and carbonyl (1654 cm^{-1}) groups. UV scanning analysis showed a strong absorption at 235 nm, which indicates the presence of a conjugated diene. As shown in Table 3, the ^{1}H- and ^{13}C-NMR spectrum revealed that the cytotoxic substance contains three methyl groups,

Table 2. Physicochemical properties of aburatubolactam C*.

Appearance	White solid
Molecular formula	$C_{30}H_{40}N_2O_5$
HRFAB-MS (*m/z*)	508.2935(M+H)$^+$
$[\alpha]_D^{20}$ (*c* 0.27, C_5H_5N)	+136°
R_f value*	0.45
Colour reaction positive	*p*-anisaldehyde, I_2
Solubility	
soluble	$CHCl_3$, DMSO, EtOAc
slightly soluble	MeOH, EtOH
insoluble	H_2O, *n*-Hexane

*Aburatubolactam C was developed on silica gel TLC plates with chloroform and diethylamine solution (7:3, v/v).

Table 3. ^{13}C-NMR and ^{1}H-NMR data of aburatubolactam C.

Pos. No.	^{13}C-NMR (100 MHz)	^{1}H-NMR (400 MHz)	Pos. No.	^{13}C-NMR (100 MHz)	^{1}H-NMR (400 MHz)
1	166.4 C		18	4.12 CH_2	3.13 (1H, m)
2	133.1 CH	6.72 (1H, d, J=14.7)			3.58 (1H, m)
3	136.1 CH	7.67 (1H, dd, J=11.8, 14.7)	19	193.1 C	
4	139.2 CH	7.49 (1H, dd, J=11.8, 15.7)	20	101.7 C	
5	134.9 CH	6.69 (1H, d, J=15.7)	21	195.1 C	
6	200.9 C		22	65.1 CH	3.60 (1H, brs)
7	65.1 CH	2.75 (1H, m)	23	26.2 CH_2	1.88 (1H, m)
8	44.0 CH	3.18 (1H)			2.12 (1H, m)
9	38.7 CH_2	0.80 (1H, m)	24	23.0 CH_2	1.52 (1H, m)
		2.02 (1H, m)			1.61 (1H, m)
10	53.1 CH	1.25 (1H, m)	25	39.7 CH_2	3.25 (1H, m)
11	48.6 CH	1.05 (1H, m)			3.70 (1H, m)
12	51.8 CH	1.72 (1H, m)	26	175.1 C	
13	41.3 CH_2	1.25 (1H, m)	27	17.6 Me	0.85 (3H, d, J=6.9)
		2.03 (1H, m)	28	26.5 CH_2	0.95 (1H, m)
14	54.0 CH	2.58 (1H, m)			1.50 (1H, m)
15	133.1 CH	5.60 (1H, dd, J=8.6, 14.8)	29	12.6 Me	0.80 (3H, t, J=6.9)
16	132.1 CH	5.50 (1H, dd, J=6.2, 14.8)	30	26.6 N-Me	2.86 (3H, s)
17	28.2 CH_2	2.53 (1H, m)	NH		9.12 1H
		2.73 (1H, m)			

NMR spectra were measured at 25°C. TMS was used as an internal reference (δ0.00). Chemical shifts are expressed in ppm and coupling constants in Hz. Pyridine-d_5 was used as solvent.

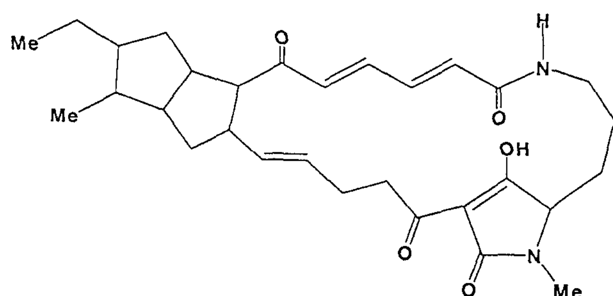

Fig. 3. Structure of aburatubolactam C.

six olefinic protons, five carbonyl groups, and double bonds in the conjugated diene. Details of the structure were determined by ^{1}H-^{1}H, ^{1}H-^{13}C COSY and HMBC spectra. These 1D and 2D NMR spectral data demonstrated that the cytotoxic substance is a novel lactam of a 20-membered macrocyclic structure coupled with a unique acyl tetramine and bicyclo[3.3.0]octane (Fig. 3).

Although the overall lactam structure of the cytotoxic substance is similar to the structure of aburatubolactam A [2], it is different in possessing 6 olefinic protons and 5 carbonyl groups, unlike aburatubolactam A which has 8 olefinic protons and 4 carbonyl groups. This novel cytotoxic substance was named aburatubolactam C.

Cytotoxic Effect of Aburatubolactam C and Apoptosis Induction

The cytotoxic effect of aburatubolactam C on several tumor cells of human and mouse origins was determined by MTT assay. Aburatubolactam C is cytotoxic for various continuously proliferating tumor cells and the IC_{50} value appeared to be 1.9 μg/ml for Jurkat, 1.2 μg/ml for U937, 1.1 μg/ml for HL-60, 6.0 μg/ml for K562, 3.9 μg/ml for COLO 320DM, 5.8 μg/ml for HepG2, 0.3 μg/ml for both BW5147.G.1.4 and P388D1, and 7.9 μg/ml for murine normal fibroblast (Table 4). Most leukemia and lymphoma cells, except K562, were sensitive to cytotoxicity of aburatubolactam C at the concentrations ranging from 0.3 to 1.9 μg/ml. However, human chronic myelogenous leukemia K562, human colon adenocarcinoma COLO 320DM and human hepatocellular carcinoma Hep G2 were less sensitive, and murine untransformed fibroblast cell line NIH 3T3 was the least sensitive to cytotoxicity of aburatubolactam C. These results suggest that cytotoxicity of aburatubolactam C is more effective on leukemias and lymphomas than on solid tumors or normal fibroblast cells.

Table 4. Inhibitory concentration of aburatubolactam C against malignantly proliferating tumor cells.

Cell line	IC_{50} (μg/ml)
Jurkat T cell	1.9
U937	1.2
HL-60	1.1
K562	6.0
COLO 320DM	3.9
HepG2	5.8
BW5147.G.14	0.3
P388DO	0.3
NIH3T3	7.9

The IC_{50} indicates a concentration of aburatubolactam C which caused 50% decrease in the number of viable cells based on MTT assay. The cells were cultured with aburatubolactam C for 2 days.

To determine the cellular mechanisms underlying the cytotoxicity, the effect of aburatubolactam C on human acute T-cell leukemia Jurkat was investigated. When cells were treated with aburatubolactam C at various concentrations of 1 to 4 μg/ml for 5 h, apoptotic DNA fragmentation was slightly detectable even in the presence of 1 μg/ml of aburatubolactam C and reached a maximal level at concentrations between 2 and 3 μg/ml (Fig. 4A), indicating that apoptotic cell death of Jurkat by aburatubolacm C is induced in a concentration-dependent manner. As shown in Fig. 4B, apoptosis of Jurkat was induced after 1 h in the presence of 3 μg/ml of aburatubolactam C and increased time-dependently. The maximal level of apoptosis was induced in 5 h and sustained until 7 h after aburatubolactam C-treatment.

Taken together these results suggest that the cytotoxic effect of aburatubolactam C is attributable to the induced apoptosis. Cellular signaling mechanisms underlying aburatubolactam C-induced apoptosis are currently being investigated in Jurkat T cells, not only to determine its potency as an antitumor agent but also to extend our understanding of the mechanisms of chemotherapy-induced apoptotic cell death.

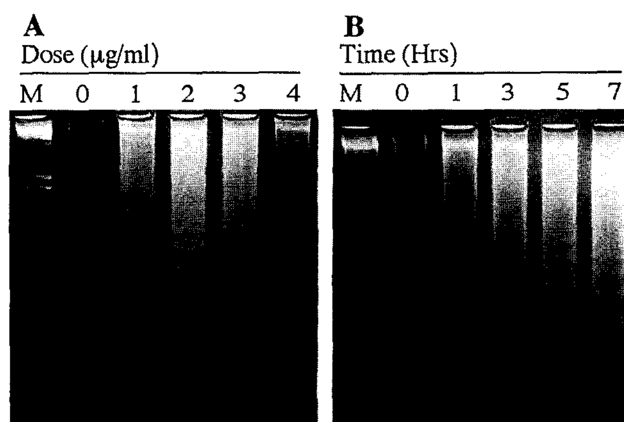

Fig. 4. Effects of aburatubolactam C on apoptotic DNA fragmentation in Jurkat T cells.

Continuously proliferating Jurkat T cells (5×10^{6}) were collected after incubation for 5 h at indicated concentration (A), and collected at the indicated times while incubated with 3 μg/ml of aburatubolactam C (B) to analyze apoptotic DNA fragmentation by NP-40 lysis method, using 2% agarose gel electrophoresis as described in Materials and Methods.

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