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Cytotoxic and Apoptotic Activites of Echinomycin Derivative (Echinomycin-7) on P388 Murine Leukemia Cells

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Echinomycin-7 is an echinomycin derivative, *S*-methylated sulfonium perchlorate of echinomycin. We studied the *in vitro* cytotoxicity and *in vivo* antitumor activity of echinomycin-7 against P388 leukemia cells and compared the results with echinomycin. With respect to the cytotoxic effects, echinomycin-7 had cell line-dependent IC_{50} values while echinomycin had similar values to several tumor cell lines. Also, *in vivo* antitumor activities were observed in tumor-bearing mice treated with both agents, which showed that echinomycin-7 had a broad therapeutic dose range. We also observed the apoptosis on leukemia cells treated with echinomycin-7 which exihibited the ladder pattern of DNA on electrophoresis. In addition to apoptosis, echinomycin-7 arrested G_1/S phases of the cell cycle at the same time. We then examined the signaling pathway of echinomycin-7-induced apoptosis and showed that ERK of the MAP kinase family was activated and translocated into the nucleus by echinomycin-7 stimulation. This study suggests that echinomycin-7 acts as an antitumor agent through *in vitro* cytotoxicity and has *in vivo* antitumor activity against leukemia cells, and that the echinomycin-7-induced apoptosis might involve signal transduction via MAP kinases.

Keywords: Antitumor activity, Cytotoxicity, DNA fragmentation, Echinomycin derivative, MAP kinase.

Introduction

Echinomycin, derived from *Streptomyces sp.*, is an antitumor agent which binds to DNA via a mechanism of bis-intercalation (Waring and Wakelin, 1974). Echinomycin is a cyclic octapeptide with two planar quinoxalines (Waring, 1992). In the clinical trials, echinomycin has been shown to possess some anti-cancer activities on ovarian and colorectal cancers (Muss *et al.*, 1990; Wadler *et al.*, 1994). However, these clinical trials have raised the need to further broaden the narrow therapeutic margin as well as to reduce the toxicity of the drug. To overcome these limitations, it was assumed that a more polar echinomycin analogue might improve its solubility and broaden the narrow therapeutic margin. Such more polar echinomycin analogues have been prepared as derivatives (Park *et al.*, 1998). Echinomycin-7 is a *S*-methylated sulfonium perchlorate derivative of echinomycin (Fig. 1). Drug-induced apoptosis is caused by nucleotide biosynthesis inhibitors and DNA-damaging agents (Kaufmann, 1989). It has been reported that camptothecin and etoposide inhibit topoisomerase I and II, respectively (Walker *et al.*, 1991) and cisplatin and actinomycin D directly damage DNA (Dyfed *et al.*, 1994; Yu *et al.*, 1996). Also, DNA fragmentation with an apoptotic response is correlated with the proteolytic cleavage of poly(ADP-ribose) polymerase during the course of chemotherapy-induced apoptosis (Kaufmann *et al.*, 1993). The relationship between drug-induced apoptosis and the cell cycle has been studied (Gorczyca *et al.*, 1993). The topoisomerase inhibitors and hydroxyurea are selectively lethal to S-phase cells, nitrogen mustard preferentially kills G_1 phase cells, and both irradiation and cisplatin selectively perturb the G_2-M phase (Barry *et al.*, 1990; Sorecson *et al.*, 1990). Also, it was reported that p38 which was activated by genotoxic chemotherapeutics was

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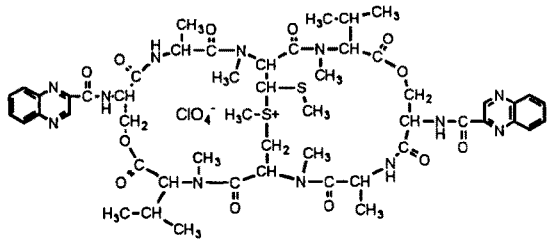

Fig. 1. Structure of echinomycin-7.

associated with a progressive inhibition of the cell cycle at G_1/S phases (Molnar *et al.*, 1997). It has been reported that the different signaling pathways were involved in apoptosis (McConkey *et al.*, 1990; Wang *et al.*, 1998). Among them, the MAP kinase family is known as an intermediate for the apoptotic signal pathway (Gardner and Johnson, 1996). Well-studied members of the MAPK family are ERK1/ERK2, which are regulated by the Ras-Raf-MEK cascade (Davis, 1993). The other member, the stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), is controlled by the Ras/Rac1/MEKK1-SEK1 cascade (Coso *et al.*, 1995). SAPK/JNK cascades were activated by DNA damaging agents such as 1-β-D-arabinofuranosylcytosine, mytomycin C, 9-nitro-camptothecin, and *cis*-platinum (Saleem *et al.*, 1995). In contrast, the other DNA-damaging agent, H_2O_2, primarily activates ERK first and later JNK (Guyton *et al.*, 1996). Also, it is known that Ras and Raf, which are the upstream components of ERK, are activated during the apoptotic signal transduction (Blagosklonny *et al.*, 1996). In this study, using the modified echinomycin derivative, its *in vitro* cytotoxicity and *in vivo* antitumor activities were evaluated. We also examined the effects of the derivative on DNA fragmentation and showed that the ERK of the MAP kinase family may be involved in an echinomycin-7-triggered apoptotic signal pathway.

Materials and Methods

Materials The preparation of the echinomycin derivative (echinomycin-7) was previously described (Park *et al.*, 1998). Phosphotyrosine antibody was purchased from Sigma Chemical Co. (St. Louis, USA). Anti-ERK antibody was purchased from Santa Cruz Biotech. Protein A-agarose was purchased from Upstate Biotechnology (Lake Placid, USA). All other chemicals and reagents were of the highest grade commercially available.

Cell culture and *in vitro* cytotoxicity assay Murine leukemia P388, murine melanoma B16, and human cervical carcinoma HeLa cells were maintained in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin, and penicillin. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. Exponentially growing cells were inoculated at 1×10^4 cells/well using 96-well plates supplemented with 100 μl DMEM. After the treated cells were incubated for 48 h, 50 μl MTT (1 mg/ml, Sigma) was added and the plates were incubated at 37°C for 4 h. To dissolve the formazan, 150 μl DMSO was added and the plates were measured at 540 nm with a spectrophotometer. The IC_{50} values were determined by plotting the logarithm of the drug concentration versus the growth rate of the treated cells.

***In vivo* antitumor activity** For evaluation of antitumor activities, P388 cells were inoculated intraperitoneally (i.p.) into BDF1 (6-wk old) mice on day 0, and the mice were divided into several groups (10 mice per group) on day 1. Compounds were diluted in normal saline and given i.p. for 7 days. Antitumor activity was expressed as the mean survival day.

DNA extraction and electrophoresis Five million cells which were treated for 48 h were washed twice in a solution of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl and then lyzed with 500 μl lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA). Lysates were harvested by $1000 \times g$ for 10 min and the supernatants were incubated for 3 h at 37°C with 50 μg/ml Rnase A, 120 μg/ml proteinase K. Then, DNAs were extracted with phenol/chloroform/isoamylalcohol (25:24:1, Sigma). After precipitation, pellets were resuspended in 30 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through a 1.8% agarose gel containing ethidium bromide and the bands visualized by UV fluorescence.

Flow cytometry After exposure for 24 h with compounds, the cells were harvested by spinning at $300 \times g$ for 5 min. The pellets were treated with Cycle TEST™ Plus DNA Reagent Kit (Becton Dickinson) and stained with propidium iodide (PI). A minimum of 2×10^5 cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, USA). Each of the G_1, S, G_2/M phases of the cell cycle was calculated using the RFIT program.

Immunoprecipitation Before cells were stimulated with compounds, they were starved for 24 h. The treated cells were lyzed in immunoprecipitation buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 50 mM β-glycerophosphate, 1% TritonX-100, 6 mM DTT, 1 mM Na_3VO_4). Lysates were centrifuged and aliquots of supernatant were incubated on ice for 4 h with phosphotyrosine antibody. Immune complexes were incubated on ice for 2 h with a 50% slurry of agarose-conjugated protein A after blocking for 1 h with 10% BSA in 50 mM NaHepes (pH 7.5). The beads were washed three times with immuno-precipitation buffer. The washed beads were boiled for 5 min in Laemmli sample buffer and the samples were rapidly spun. Finally, the supernatant was used for SDS-PAGE and immunoblot analyses.

Preparation of nuclear extracts All the fractional procedures were completed on ice. The treated cells were washed twice and collected into 1 ml hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 40 μg of PMSF per ml and 10 μg of both pepstain and leupeptin per ml, pH 7.5). The cellular suspension was homogenized and harvested by centrifugation at $600 \times g$ for 5 min. To prepare the cytosolic fraction, the supernatant was centrifuged at $12{,}000 \times g$ for 20 min. The pellets

were resuspended in 0.25 M sucrose in 1.5% citric acid and passed three times through a 26 gauge needle and finally loaded onto 1 ml of 0.88 M sucrose cushion in 1.5% citric acid. To obtain the nuclear fraction following centrifugation at 900 × *g* for 10 min, the pellets were dissolved with hypotonic lysis buffer containing 0.5% Igepal CA-630, 0.1% deoxycholate, 0.1% Brij-35, and then centrifuged at 10,000 × *g* for 10 min. The purity of nuclear fraction was monitored by measuring the lactate dehydrogenase activity as the cytosol marker. Then, the nuclear fractions were confirmed by Western blot analysis.

Results and Discussion

***In vitro* cytotoxic effect and *in vivo* antitumor activity of echinomycin-7** Echinomycin was known to be a very toxic antitumor agent, the therapeutic range of which was difficult to determine *in vivo*. To reduce the toxicity, other studies, like the preparation of tumor-specific immunoliposomes containing doxorubicin, have been carried out (Nam *et al.*, 1998). Also, the effects of noncytotoxic concentrations of anticancer drugs on doxorubicin cytotoxicity were tested (Lee and Lee, 1996). In this study, we compared the properties of echinomycin-7 with echinomycin. The cytotoxic activities were evaluated by the MTT method against several tumor cell lines and the IC_{50} values of these compounds were calculated (Table 1). Echinomycin-7 had a cell line-dependent IC_{50} range between 14.46 and 65.01 nM, but in the case of echinomycin, the range narrowed from 3.94 to 5.02 nM. Cell growth inhibition by echinomycin-7 exhibited the characteristic dose-response curves against different cell lines (Fig. 2). In conclusion, echinomycin-7 had a less cytotoxic effect than echinomycin. The *in vivo* antitumor activities of echinomycin and echinomycin-7 were examined in P388 leukemic mice. As shown in Table 2, the mean survival day was prolonged in the groups treated with echinomycin and echinomycin-7 compared with the control groups. Echinomycin-7 showed the more effective antitumor activity with a broader therapeutic range. Thus, we speculate that echinomycin-7 is more effective against leukemia than echinomycin and less toxic *in vivo*.

Table 1. MTT assay for IC_{50} values of echinomycin and echinomycin-7 on various cell lines.

	IC_{50}[a]	
Cell line	Echinomycin	Echinomycin-7
P388	5.02 nM	46.48 nM
B16	3.94 nM	14.46 nM
HeLa	4.96 nM	65.01 nM

[a] IC_{50} (50% inhibitory concentration) is equal to the compounds' concentrations when sample absorbance−blank absorbance/control absorbance−blank absorbance × 100 is 50.

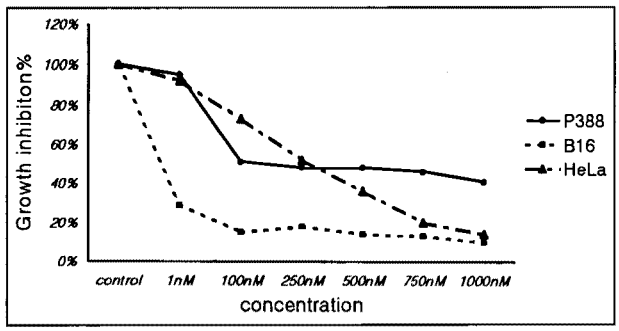

Fig. 2. Growth inhibition of three cell lines after exposure to echinomycin-7 for 48 h. Points represent the means of at least four replicate experiments.

DNA fragmentation We examined the apoptotic response as judged by the appearance of a DNA ladder upon gel electrophoresis at various concentrations of echinomycin and achinomycin-7 (Fig. 3). It was shown that echinomycin-7 induced apoptosis at concentrations between 50 nM and 500 nM, and the ladder was also observed at 1 μM of treatment (data not shown). On the contrary, only 500 nM of echinomycin showed apoptosis. Thus, while leukemia cells were induced to undergo apoptosis by both echinomycin-7 and echinomycin, echinomycin-7 exhibited DNA fragmentation in a broader range of concentrations.

Cell cycle analysis It has been known that most antitumor drugs which induce apoptosis cause cell cycle arrest, but the relationship between apoptosis and cell cycle arrest remains to be explored. Recently, it was reported that the apoptotic signal pathway is related to the arrested phase of the cell cycle (Cortes *et al.*, 1997; Molnar *et al.*, 1997). In order to analyze the effect of echinomycin-7 on the cell cycle, the treated cells were labeled with propidium iodide

Table 2. *In vivo* antitumor activity of echinomycin-7 on P388 leukemia-implanted mice.

	N[a]	i.p. (mg/kg)[b]	T/C(%)[d]
Control[c]	10		100
Echinomycin-7	8	1	135.5
	8	0.25	130.7
	6	0.0125	130.6
	6	0.0625	127.3
	6	0.03125	120.3
Echinomycin	8	0.025	129.3

[a] N: number of mice

[b] Mice (BDF1) were implanted intraperitoneally (i.p.) with tumor cells (day 0) and the drug was administered (mg/kg) intraperitoneally (i.p.) everyday (days 1–9).

[c] Control: tumor-bearing mice without antitumor drug.

[d] T/C (%) = (the mean survival day of treated mice/the mean survival day of control mice) × 100.

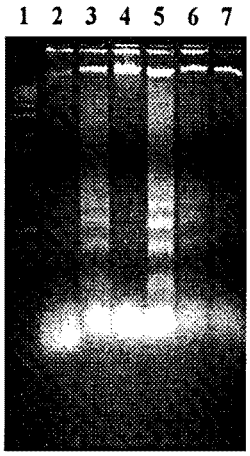

Fig. 3. DNA fragmentation of P388 cells by treatment with 50 nM, 500 nM, 5 μM echinomycin (lanes 2–4), and 50 nM, 500 nM, 5 μM echinomycin-7 (lanes 5–7). Lane 1, 1 kbp ladder marker. Cellular DNAs were extracted and electrophoresed on a 1.8% agarose gel to detect DNA fragmentation.

(PI) and analyzed by flow cytometry (FACSan). The DNA peaks of P388 cells exposed to the various concentrations of drugs were examined (Fig. 4). The results were that 500 nM of echinomycin-7 arrested cells in G_1, but 1 μM and 2 μM compounds resulted in S phase arrest. Thus, echinomycin-7 inhibits cell cycle progression by arresting the G_1/S phase of the cell.

Activation and translocation into nucleus of MAP kinase (ERK2) It is known that the MAPK pathway is involved in apoptotic signal transduction (Wang *et al.*, 1998). To determine if ERKs, a member of the MAPK family, were activated by echinomycin-7 to induce an apoptotic response, we examined the phosphorylation of ERKs by immunoblot analysis (Fig. 5A). P388 cells were exposed to 500 nM of echinomycin-7 for 10, 30, and 60 min. It is known that MAP kinases are phosphorylated on both tyrosine and threonine residues in response to various stimulations (Boulton *et al.*, 1991). First, we separated the tyrosine-phosphorylated proteins from the cellular extracts by immunoprecipitation and the activation of ERKs was confirmed by immunoblot analysis using anti-ERK antibodies. The result was that echinomycin-7 activated the 42 kDa ERK2 at 30 and 60 min after treatment. It has been reported that the activation of ERK is required for its translocation into the nucleus (Chen *et al.*, 1992). To confirm this nuclear translocation, we examined the separation of nuclei from the cells exposed with echinomycin-7 for 10, 30, and 60 min and performed an immunoblot analysis (Fig. 5B). Our result showed that the ERK2 (42 kDa) appeared to be in the nuclear fraction at 30 min, but it disappeared at 60 min. We had previously shown that ERK2 (42 kDa) translocated to the nucleus at 30 min by echinomycin stimulation (Kim *et al.*, 1996). As

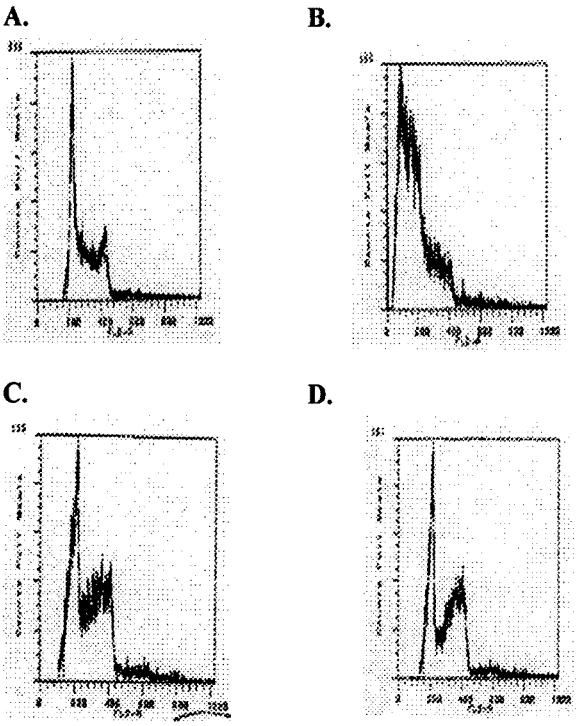

Fig. 4. Cell cycle analysis of P388 cells exposed to echinomycin-7 by flow cytometry. A. Untreated cells (G_1, 32.8%; S, 52.5%; G_2 + M, 14.7%). B. 500 nM exposed cells (G_1, 64.2%; S, 15.4%; G_2 + M, 20.4%). C. 1 μM exposed cells (G_1, 11.0%; S, 80.9%; G_2 + M, 7.8%). D. 2 μM exposed cells (G_1, 22.2%; S, 68.2%; G_2 + M, 9.6%).

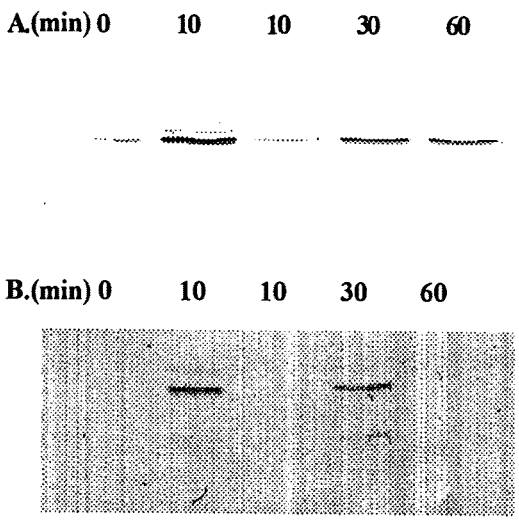

Fig. 5. Immunoblot analysis of phospho-MAP kinase and ERK translocation to nucleus. The starved P388 cells were treated with echinomycin-7 for 10, 30, 60 min (lanes 3–5), serum-free cellular fraction (lane 1), and serum-added fraction (lane 2). A. After phosphotyrosin protein were immunoprecipitated by anti-phosphotyrosin antibody, Westen blot analysis was performed using anti-ERK antibody. B. Western blot analysis using anti-ERK antibody in the nuclear fraction.

shown in Fig. 5, ERK2 (42 kDa) was phosphorylated and translocated into the nucleus by echinomycin-7 under the same conditions for inducing apoptosis. Accordingly, ERK2 may be involved in the signal transduction of echinomycin-7-induced apoptosis.

In summary, echinomycin-7 has antitumor activity with a broad range of effective dose against leukemia and induces an apoptotic response with G_1/S phase arrest. Also, our results imply that the activation and translocation of ERK2 into the nucleus may be related to the apoptotic signal transduction of echinomycin-7.

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