

The Role of MAP Kinase Pathways in Echinomycin and Novel Analogue-induced Apoptosis of HT-29 Cells

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Echinomycin, typical DNA minor groove binder had comparable efficacy compared to 5-FU in phase II trial for colon cancer treatment. To improve echinomycin's drawback, we synthesized YK-2000 series (echinomycin analogues). Echinomycin and YK-2000 enabled to induce the apoptosis on HT-29 colorectal cancer cell line. Apoptosis in HT-29 cell triggered by echinomycin and YK-2000 were proven through DNA laddering, PARP (poly-(ADP-ribose) polymerase) cleavage and flow cytometric analysis. Such an apoptotic pattern displayed by YK-2000 was quite different from that of echinomycin. Next, to explore signaling pathway of echinomycin and YK-2000, we examined phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 MAP kinase. However, it is not clearly that the mechanism of cancer cell death induced by echinomycin and YK-2000. Here, we report that one of major apoptotic signaling pathway induced by echinomycin and YK-2000 may be MAP kinase pathway in HT-29 human colon cancer cells.

Key Words: Echinomycin, YK-2000, Apoptosis, MAP kinase, PARP

INTRODUCTION

There is a growing body of evidence from pre-clinical and clinical studies that synthetic or natural DNA damaging agents are able to induce apoptosis of human colon cancer cells.^{1,2)} Among these, minor groove binder has attracted attentions for DNA targeting therapeutics for colon cancer chemotherapy.^{3,4)}

Of minor groove binders, echinomycin, one of prototype DNA bisintercalator earned comparable efficacy to that of 5-FU in the clinical trials to target colon cancer patients.^{5,6)} However, mechanism involved in cytotoxicity of echinomycin on colon cancer cell was unclear. Previously we have shown that echinomycin exerted potent cytotoxicity on human colon cancer cell lines.⁷⁾ As a possible mechanism of

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potent cytotoxicity, we first reported that echinomycin enabled to induce apoptosis in murine leukemic cells *in vitro*.⁸⁾ Moreover, we demonstrated that methyl sulfonium derivative of echinomycin had an equivalent or better activity in mouse leukemic and melanoma model.⁹⁾ However, the mechanism of echinomycin beside classical bisintercalation into tumor DNA base was unknown.³⁾ Currently, we presented the evidence that echinomycin and its derivative may trigger MAP kinase pathway in mouse leukemic cells, leading to synergistic apoptosis.⁹⁾

Likewise, antitumor agent-induced apoptosis is occurred by different mechanism such as the nucleotide biosynthesis inhibition,¹⁰⁾ signal transduction inhibition and direct DNA damage.¹¹⁾ It has been well-documented that different signaling pathway such as MAP kinase, Ras and caspase pathway were involved with antitumor agent-induced apoptosis.¹²⁾ Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) is controlled by Ras/Rac 1/MEKK-SEK1 cascade.^{13,14)} SAPK/JNK cascades were activated by DNA damaging agents such as mitomycin C, 9-nitro-camptothecin and cis-platinum.^{15,16)} Of several signal pathways, we hypothesized that MAP kinase pathway would be strongly involved with apoptotic phenomena in solid tumor cells when treated with echinomycin. Since Ech-7, echinomycin derivative was developed in 1998⁹⁾ another improved analogue YK-2000 has been available.

Recent reports demonstrated that one of the early reactions occurring in the cell after the damage of its DNA is the poly-(ADP-ribose)polymerase (PARP) and that PARP is involved in different cellular functions including DNA repair, DNA replication, and programmed cell death. Cleavage of PARP leads to an activation of Ca^{2+}/Mg^{2+} dependent endonuclease¹⁷⁻¹⁹⁾ involved in DNA fragmentation in apoptosis. Internucleosomal DNA fragmentation is a characteristic event in apoptotic death.¹⁸⁾ Caspases are known to cleave several structural and regulatory proteins, such as poly (ADP-ribose) polymerase (PARP), lamine, and DFF45. Specifically, an involvement of caspase-3 has

been proposed in DNA fragmentation shown during apoptosis.¹⁸⁾

In the present study, we investigated the impact of echinomycin and YK-2000 treatment on the activity of the MAP kinase pathway in the regulation of responses and the characterization of the mechanism by which drug induces apoptosis. Here we report that triggering of MAP kinase pathway activation through echinomycin and YK-2000 treatment may induce apoptosis of HT 29 cells.

MATERIALS AND METHODS

1) Materials

YK-2000 was provided by Dr. Y. H. Kim (KAIST, Korea). RPMI-1640 and Fetal Bovine Serum (FBS) were purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Reagents for SDS-PAGE and protein determination were purchased from Bio-Rad (Richmond, CA). Antibody to phospho-SAPK/JNK, phospho-ERK1/2 and phospho-p38 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Poly-ADP-ribose polymerase (PARP) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz CA). Peroxidase-labelled goat anti-rabbit IgG and anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Annexin V-FITC kit was purchased from Clontech Laboratories, Inc. (East Meadow circle Palo Alto, CA). All the other chemical and reagents were the highest grade and commercially available.

2) Cell lines and cell culture

Human colon cancer cell line HT-29 were purchased from American Type Culture Collection (ATCC) (Rockville, MD). HT-29 cells were routinely maintained in RPMI-1640 supplemented with 10% FBS, streptomycin and penicillin. Cells were cultured at 37°C in a humidified atmosphere of CO₂ and the medium was changed every two days.

3) Cell viability assay

Drug effect on cellular viability was evaluated using an assay based on the cleavage of the yellow dye MTT to purple formazan crystals by dehydrogenase activity in mitochondria, a conversion that occurs only in living cells. One hundred μ l of cell suspensions (2×10^4 cells/well) were put into each well of 96-well flat-bottomed microtiter plates, and each plate was incubated for 24 h at 37°C and 5% CO₂ atmosphere. After incubation, 100 μ l reagent solutions or media at the desired concentrations were distributed into each well. The microtiter plates were incubated for 72 h. There after, 20 μ l of the MTT dye (5 mg/ml, sigma) were added and the plates were incubated at 37°C for 4 h. To dissolve formazan, 150 μ l DMSO was added. The plates were subjected to measurement at 540 nm by spectrophotometer. IC₅₀ values were determined by plotting the logarithm of the drug concentration versus the growth rate of the treated cells.

4) DNA fragmentation

Various concentrations of echinomycin or YK-2000 were added to 5×10^6 HT-29 cells and incubated for 24 h. Cells were washed twice in a solution of ice cold PBS and harvested by centrifugation at 14,000 rpm for 10 min. The pellets were lysed with 500 μ l lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA) in 1 h on ice. Lysates were harvest by 14,000 rpm for 10 min and supernatants were collected and incubated for 3 h at 55°C with 50 μ l proteinase K (1 mg/ml), 10 μ l RNase A (10 mg/ml), and 10 μ l 10% SDS. Then, DNA was extracted by phenol : chloroform : isoamylalcohol (25 : 24 : 1). After ethanol precipitation, pellets were resuspended in 30 μ l TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed thorough a 1.8% agarose gel contained with Ethidium bromide (0.5 μ g/ml) and gel was visualized by UV fluorescence.

5) Apoptosis assessment by annexin-V/propidium iodide staining

Apoptosis in HT-29 cells were measured by annexin-V/propidium iodide kit. Briefly, after drug treatment the cells were washing in PBS and resuspended in 400 μ l binding buffer (containing annexin-V fluorescein and propidium iodide). After incubation at room temperature for 30 min, cells were analyzed by flow cytometry (Becton Dickinson, NJ).

6) Immunoblot analysis

Test cells were washed with ice-cold PBS and lysed in ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM PMSF, 1 mM NaF. And then, the cell suspension was kept on ice for 30 min. The lysate was cleared by centrifuge at 15,000 rpm for 10 min at 4°C. The lysate was resolved by SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. After blocking, membranes were incubated at 37°C for 1 h with a primary antibody (1 : 1000) and for 1 h with the corresponding secondary horseradish peroxidase-conjugated secondary antibody (1 : 2000). The membrane was then developed in ECL reagent and exposed to x-ray film.

RESULTS

1) *In vitro* cytotoxic effect of YK-2000

Cytotoxicities were evaluated by MTT method

Table 1. Cytotoxic effect measurement of echinomycin and YK-2000 on various cell lines by MTT assay

Cell line	IC ₅₀ ^a		
	Echinomycin	YK-2000	ADR ^b
HT-29	0.0024 μ g/ml	0.156 μ g/ml	0.156 μ g/ml

^aIC₅₀: Inhibition concentration

^bADR: Adriamycin

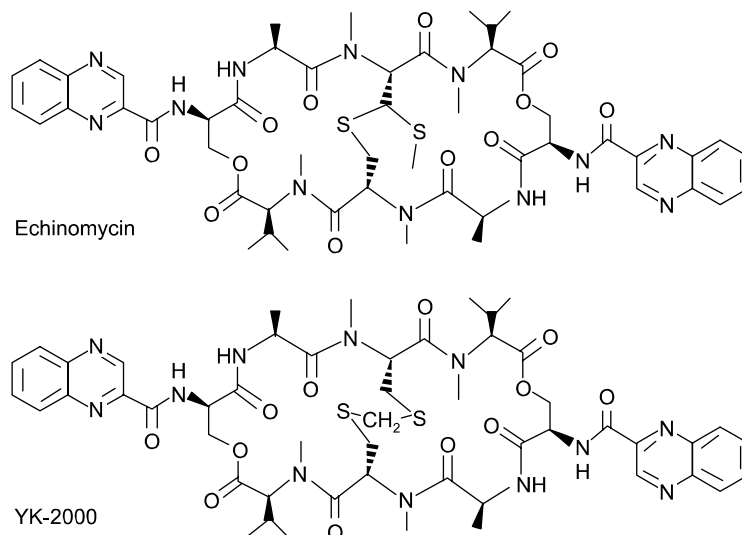


Fig. 1. Chemical structures of echinomycin and YK-2000.

against HT-29 cancer cell line, IC_{50} values of test compounds were calculated. IC_{50} value of YK-2000 on HT-29 cell is $0.156\mu\text{g/ml}$. IC_{50} value of echinomycin was 80 fold lower than that of YK-2000 (Table 1).

2) DNA fragmentation induced by echinomycin and YK-2000

To define dose-response and time kinetics in HT-29 colon cancer cell line treated with echinomycin and YK-2000. HT-29 cells were exposed to the escalating concentrations of echinomycin or YK-2000 for 24 h and subjected to DNA fragmentation. DNA laddering was typically appeared on HT-29 cells when treated with echinomycin ($0.2\mu\text{g/ml}$) for 24 h (Fig. 2A). In parallel PARP cleavage was clearly shown in HT-29 cells, which were treated with various dose ($0.2\sim 10\mu\text{g/ml}$) of echinomycin during 24 h (Fig. 2B). Next, we examined the time kinetics of DNA fragmentation with echinomycin ($2\mu\text{g/ml}$). DNA fragmentation was detected after 6 h of treatment with echinomycin (Fig. 2C). Immunoblot for PARP cleavage revealed the cleavage of 85 kDa fragment from whole PARP after echinomycin treatment (Fig. 2D). Meanwhile, increase

concentration of YK-2000 induced apoptotic DNA fragmentation (Fig. 3A). Apoptotic DNA fragmentation was detected after 24 h of treatment with YK-2000 ($10\mu\text{g/ml}$)(Fig. 3C). To confirm the cleavage of endogenous nuclear protein PARP in apoptotic cells, immunoblot was performed with anti-PARP antibody (Fig. 3B and 3D), corresponding to intact PARP cleavage in YK-2000 treated cells. PARP cleavage were increased by treatment of YK-2000, indicating apoptosis induced by YK-2000.

3) Effect of echinomycin and YK-2000 on cell death

The annexin-V-FITC binding assay was performed to confirm the degree of apoptosis in echinomycin or YK-2000 mediated cell death. Annexin-V binds to phosphatidylserine-exposed cells on the outer membrane of cell, while propidium iodide stains DNA of cells with the damaged cell membrane. Echinomycin and YK-2000 definitely produced a number of death cells, (25% and 16%) following 19h-exposure to YK-2000 ($10\mu\text{g/ml}$) and echinomycin ($2\mu\text{g/ml}$) respectively (Fig. 4). Apoptosis inducibility of YK-2000 was likely more potent in terms of IC_{50} and drug

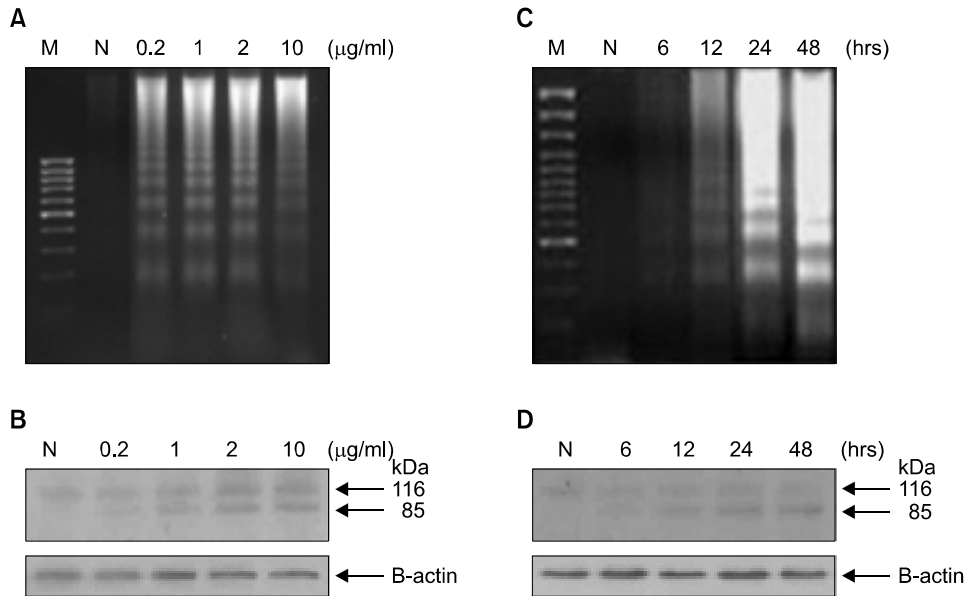


Fig. 2. Induction of internucleosomal DNA fragmentation by echinomycin in HT-29. (A) HT-29 cells were cultured in media containing echinomycin at the indicated concentrations. HT-29 were treatment with echinomycin for the indicated time (24 hrs), after which total DNA was extracted and analyzed by agarose gel electrophoresis (M: DNA marker, N: naive HT-29). (B) Cleavage of 116 kDa PARP into an 85 kDa apoptotic fragment was monitored by immunoblot analysis. HT-29 cells were cultured with the indicated concentrations of echinomycin. After 24 h, cells were collected and analyzed for PARP cleavage. (C) Cells were cultured in media containing echinomycin (2µg/ml). At the indicated time points, cells were harvested and lysed. DNA fragmentation was examined by agarose gel electrophoresis (M: DNA marker, N: naive HT-29). (D) Cleavage of PARP after treatment with echinomycin (2µg/ml) for indicated time.

concentration inducing apoptosis compared to echinomycin.

4) Echinomycin activates MAP kinase

HT-29 cells were treated with echinomycin (2µg/ml) at regular time interval (10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h). Using phospho-specific antibodies against MAP kinase, ERK1/2 phosphorylation began quickly following echinomycin treatment and reached plateau at last 24 h. Meanwhile, SAPK/JNK was activated 10 min after echinomycin treatment and then the kinase activity was decreased. Contrary to p38 activation by YK-2000, phosphorylation of p38 began at 10~30 min after echinomycin treatment and reached maximum peak at 4 h (Fig. 5).

5) Effect of YK-2000 on MAP kinase activation

SAPK/JNK is part of a kinase cascade, which is strongly stimulated by various DNA-damaging agents. To confirm MAP kinase activation by YK-2000, HT-29 cells were treated with YK-2000 (10µg/ml) and the phosphorylation status of three MAP kinase subfamilies (ERK1/2, p38 and JNK) in apoptotic cells were analyzed by immunoblotting with phospho-specific antibodies. Surprisingly, ERK1/2 activity was peaked at 2 h after treatment of YK-2000. The SAPK/JNK and p38 activity after exposure of HT-29 cells to YK-2000 markedly decreased in activity and began at 4 to 8 h post treatment of YK-2000. Two MAP kinase (SAPK/JNK and p38) activation step

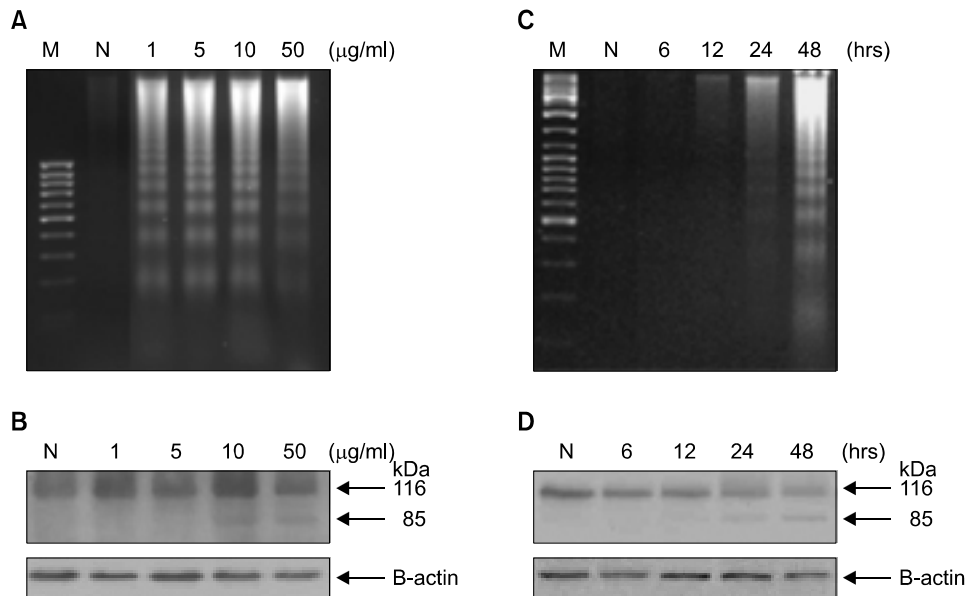


Fig. 3. YK-2000 induced DNA fragmentation in HT-29 cells. (A) Cells were cultured in media containing YK-2000 at the indicated concentrations (for 24 hrs) (M: DNA marker, N: naive HT-29). (B) Cleavage of 116 kDa PARP into an 85 kDa apoptotic fragment indicated concentration of YK-2000 (for 24 hrs). (C) Cells were cultured in media containing YK-2000 (10µg/ml). At the indicated time points, cells were harvested. Purified DNA was separated by electrophoresis in 1.8% agarose gel at 100 V for 60 min. Ethidium bromide staining and UV illumination were used to visualize DNA fragmentation. (D) Cleavage of 116 kDa PARP into an 85 kDa apoptotic fragment after treatment with YK-2000 (10µg/ml) for indicated time.

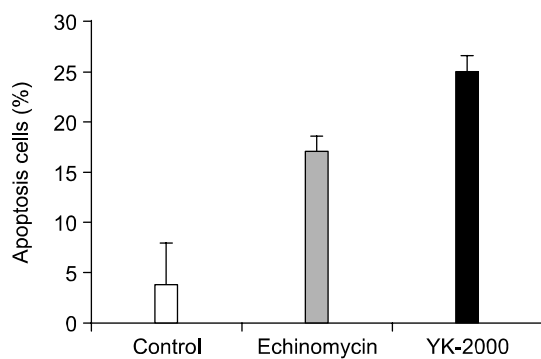


Fig. 4. Percentage of apoptosis in HT-29 cells treated with YK-2000 or echinomycin. HT-29 cells were treated with echinomycin (2µg/ml) or YK-2000 (10µg/ml) and incubated for 24 h. Results of flow cytometric analysis are presented as the mean±SE of two experiments.

forward with roughly similar time courses (Fig. 6).

DISCUSSION

Echinomycin is a novel antibiotic with diverse activity such as, DNA bisintercalation,^{20,21)} CG base specific binding,⁵⁾ potent cancericidal activity,²²⁾ tissue factor suppression²³⁾ and enhancement of peptide nucleic acid binding.²⁴⁾ To date, we proved that echinomycin is a strong apoptosis-inducer on mouse leukemia cells.⁹⁾ Despite definite apoptosis inducibility on cancer cells by echinomycin, there is no clear evidence whether echinomycin, its derivative or analogue triggers the ERK1/2, JNK or p38 kinases pathway or whether these compounds contribute to its apoptosis via MAP kinase pathway. In addition, the impact of echinomycin on human solid cancer cells

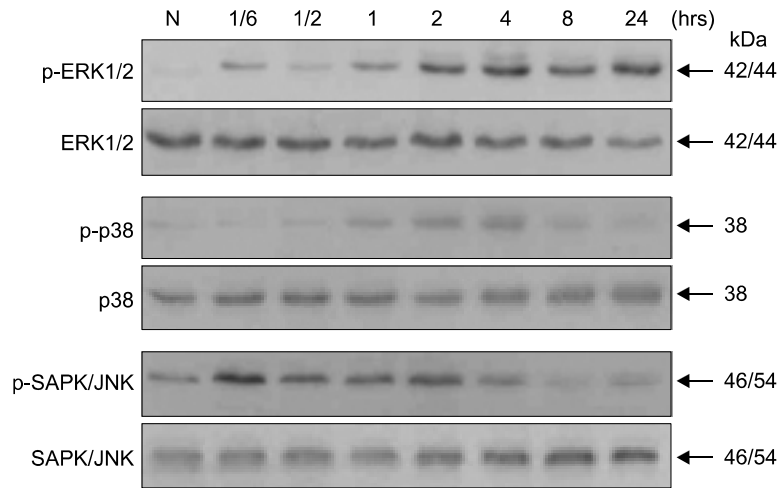


Fig. 5. Time-dependent changes in activities of ERK1/2, SAPK/JNK and p38 in HT-29 cells after exposure to echinomycin (2 μ g/ml). Immunoblots were probed with antibodies specific for total protein or activated phosphorylation form of ERK1/2, SAPK/JNK, p38.

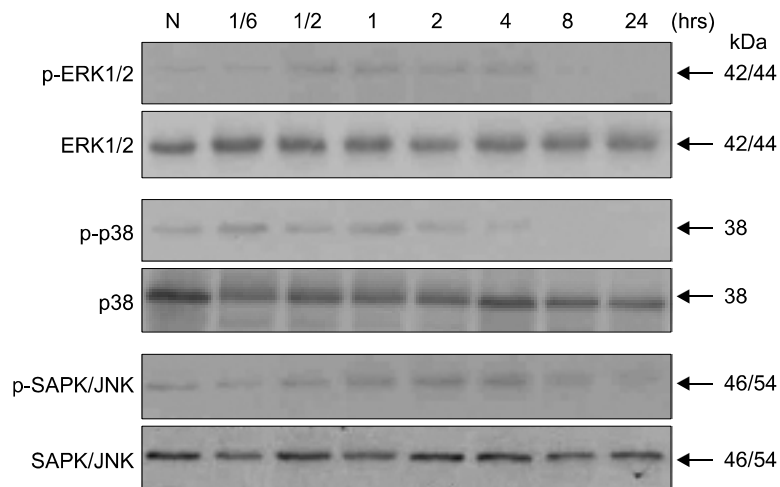


Fig. 6. Time-dependent activation of ERK1/2 and reduction of SAPK/JNK and p38 by treatment of YK-2000 (10 μ g/ml) in HT-29 cells. Immunoblotting of phosphorylated ERK1/2, SAPK/JNK and p38.

was unknown. In the present study, we clearly demonstrated apoptosis on HT-29 colon cancer cell line with echinomycin and YK-2000, the echinomycin analogue as well as the role of MAP kinase pathway leading to apoptosis. YK-2000 was designed to circumvent echinomycin's hydrophobicity as well as to attenuate immune cell toxicity. Thus, we should not only expected the decreased cytotoxicity on cancer

cells but also more effective eradication of human colon cancer cells via induction of apoptosis. Augmenting apoptosis through signaling pathway is essential to apoptosis-inducing antitumor agents.²⁵⁾ Apoptosis inducibility by YK-2000 was likely more potent than parent molecule, echinomycin.

Therefore, despite absence of in vivo study using YK-2000, our results warrant additional studies on the

therapeutic activity of YK-2000 *in vivo*. It should be pointed out that YK-2000 inhibited cell growth or decreased cell survival as shown in Table 1.

Most cancer chemotherapeutic agents induce apoptosis. However, signal transduction mechanisms regulating apoptosis was not clearly defined, although proline-directed serine/threonine kinases of MAP kinase family were strongly implicated. Recent studies have shown that bisintercalator such as actinomycin, induce apoptosis in several types of solid cancers including human pancreas cancer cell line.²⁶⁾ Various chemically and biologically different agents have been found to mediate the induction of apoptosis through activation of p38.²⁷⁻³⁰⁾ Likewise, SAPK/JNK has been predominantly associated with stress responses such as apoptosis and cytokine release.^{31,32)} During apoptosis process triggered by YK-2000, phosphorylation of ERK1/2 was increased, whereas phosphorylation of SAPK/JNK and p38 were downregulated in comparison to control cell. These results suggest that activation of ERK1/2 as well as downregulation of SAPK/JNK and p38 might mediate YK-2000 induced apoptotic process. Interestingly, MAP kinase pattern displayed by echinomycin and YK-2000 was similar but p38 phosphorylation is specifically different. In the naive colon cancer tissues, it was reported highly activation of SAPK/JNK and p38.³³⁾ In contrast to this pattern, SAPK/JNK and p38 were downregulated in apoptotic cells induced by YK-2000. This result is not consistent with other apoptosis models in which SAPK/JNK and p38 activation was crucial for apoptosis signaling. Such results suggest that higher SAPK/JNK and p38 activity is not required for induction of apoptosis in HT-29 cells by treatment of echinomycin and YK-2000. These and previous data support that cross-talk between ERK1/2 and p38 kinase signaling determines apoptosis.^{34,35)} However, despite divergent role of two different signaling, it remained to be seen if which kinase would be survival or death signal in apoptosis induced by DNA damaging-bisintercalator such as echinomycin.

Another important implication in this study is that

cell death induced by bisintercalator may be mediated through MAP kinase pathway besides DNA binding. Based on these findings, we suggest that YK-2000 may be a good candidate for additional evaluation as a cancer therapeutic agent for human colon cancer as well as other types of solid cancer implicated with aberrant expressions of MAP kinase.

These results indicate that echinomycin and novel synthetic echinomycin analogue may modulate MAP kinases activation in human HT-29 cell line.

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