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DNA Bis-intercalating Agent, Echinomycin-induced Apoptosis via Bcl-2 Dependence Pathway in Human Colon Cancer Cells

Ju Youn Park[1,2], Yong Suk Ryang[2], Jong Bae Kim[2], Jae-Ho Chang[3], Hyeon Cheol Cho[1] & Soo-Ki Kim[1,3]

[1]Department of Microbiology, Yonsei University Wonju College of Medicine, Wonju 220-701, Korea
[2]Department of Biomedical Laboratory Science, College of Health Science, Yonsei University, Wonju 220-710, Korea
[3]Institute of Lifelong Health, Institute of Basic Medical Science, Yonsei University Wonju College of Medicine, Wonju 220-701, Korea
Correspondence and requests for materials should be addressed to S. K. Kim (kim6@yonsei.ac.kr)

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Abstract

Despite versatile activity (cancericidal, antimicrobial, hypoxia inducible factor (HIF) inhibition, immune deactivation of DNA bis-intercalation agent, echinomycin, its specific mechanism has been elusive. Of these novel mechanisms, we reported that using human colon cancer cells (HT-29), apoptotic machinery induced by echinomycin might be dependent of caspase-3 pathway. Despite a partial enlightenment of prototypic signal path triggered by echinomycin, the role of Bcl-2 in this signaling pathway is unclear. To address this issue, we explored whether or not echinomycin would overcome the anti-apoptotic impact of Bcl-2 in HT-29 cells by the controlled Bcl-2 overexpression. Prior to this proof, we confirmed that echinomycin induces mitochondrial depolarization, then triggering the mitochondrial pathway of apoptosis with an involvement of upstream caspases-3. Transiently transfection with inactive Bax-DNA failed to prevent echinomycin-induced apoptosis in HT-29 cells. To dissect the role of Bcl-2 in echinomycin-induced apoptosis, HT-29 cells were transiently transfected with Bcl-2 DNA for overexpression and then treated with echinomycin for 24 h. Combined analyses of DNA fragmentation and flow cytometric analysis clearly verified that echinomycin -induced apoptosis was drastically attenuated by Bcl-2 overexpression, whereas a control vector rarely affected echinomycin-induced apoptosis. Collectively, these data verify that Bcl-2 regulates echinomycin-induced apoptosis in HT-29 cells. To my knowledge, this is the first evidence that of diverse, structured minor groove binders (MGB), the prototypic echinomycin might control the apoptotic signaling via Bcl-2-mitochondrial pathway.

Keywords: Apoptosis, Echinomycin, Bcl-2, Cytochrome *c*, HT-29 cell

Echinomycin was classically known as DNA bis-intercalating or DNA damaging-quinoxaline molecule. Emerging evidences have shown that echinomyin or DNA bis-intercalator owns novel medicobiologic activities such as HIF suppression, transactivator of transcription (TAT) [of human immunodeficiency virus (HIV)] binding, anti-vancomycin-resistant enterococci (VRE) activity, and anti-thrombotic activity[1-4]. Such diversified activities are unexplainable only by simple notion of echinomycin's DNA targeting, suggesting that echinomycin might modulate cellular machinery involving apoptosis and differentiation in prokaryotes or eukaryotes. Hypothesizing this, we have presented evidences that echinomycin triggers intracellular signaling pathways such as the mitogen-activated protein kinases (MAPKs), mitochondrial, and caspase pathways, consequently leading to apoptosis of HT-29 cells[5,6]. However, previous experiment had clear limitations in terms of demonstrating the engagement of Bcl-2 in echinomycin-mediated apoptosis[6].

It was widely accepted that apoptosis via the mitochondrial pathway induces mitochondrial outer membrane permeabilization (MOMP), then leading to the release of cytochrome *c*. This key step toward apoptosis is mediated by proteins of the Bcl-2 family[7]. Compelling data have shown that Bcl-2 overexpression inhibits apoptosis induced by diverse anticancer drug[8]. Despite a partial identification of prototypic signal path triggered by echinomycin, the role of Bcl-2 in this signaling pathway is unclear.

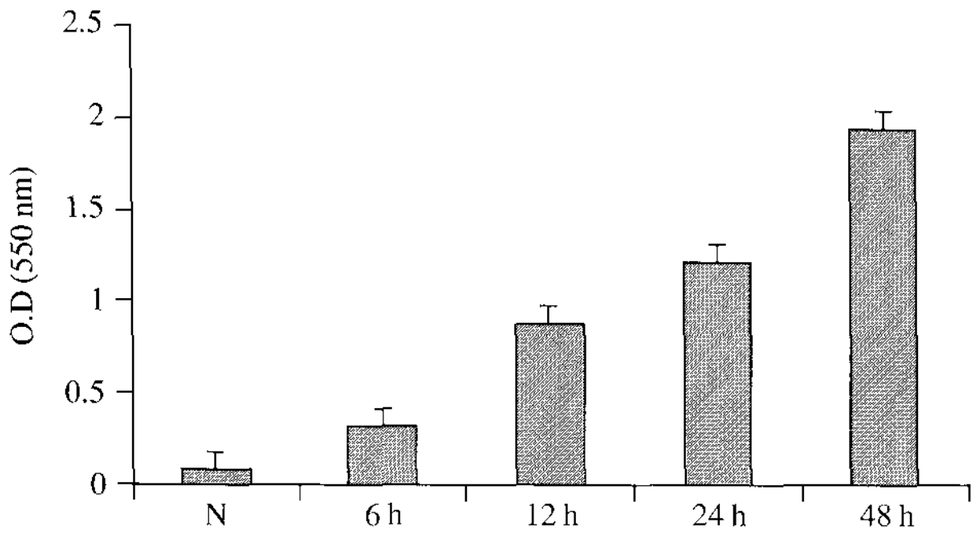


Figure 1. Echinomycin-induced apoptosis in HT-29 cells. Echinomycin was incubated with HT-29 cells induce apoptosis for indicated time. After the echinomycin treatment (2 μg/mL), cells undergoing apoptosis were detected by the uptake of a purple dye (APOPercentage™ Apoptotic Assay, Biocolor). Data are presented as means ± S.D. of 3 separated experiments.

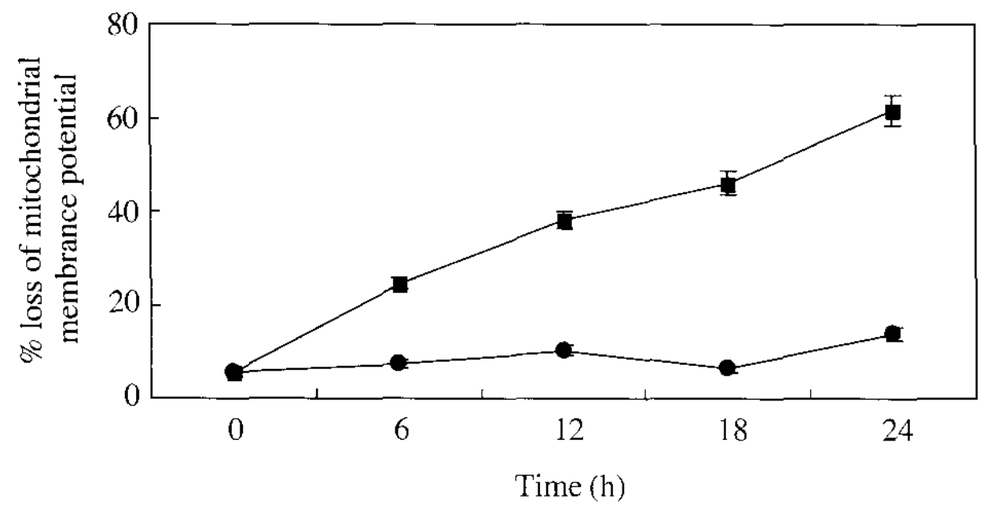
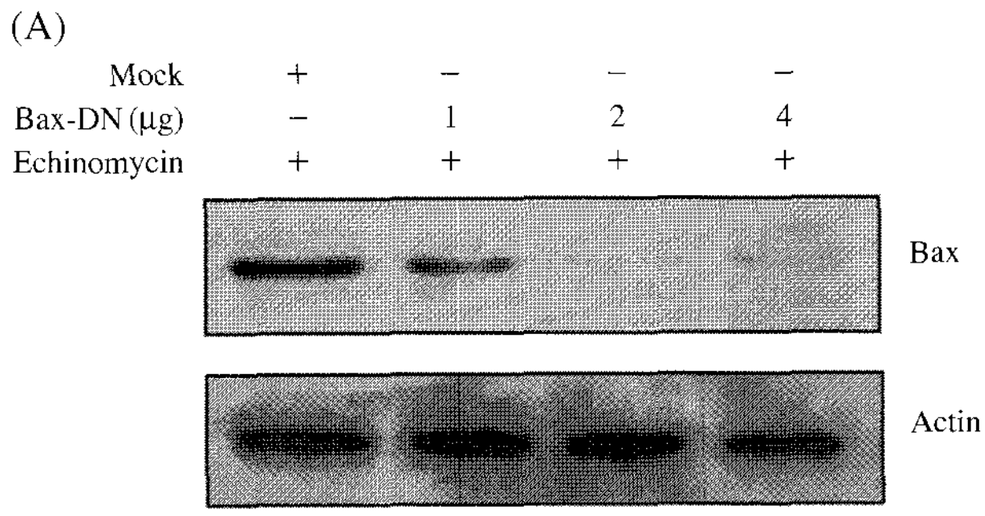


Figure 2. Mitochondrial membrane potential in echinomycin-treated cells. HT-29 cells were untreated (control, circles) or treated with 2 μg/mL echinomycin (squares) for the times indicated. The cells were then stained with JC-1, and the cells with intact mitochondrial membrane potential were scored. The percentage of cells with intact membrane potential is plotted as a function of time. Data are presented as means ± S.D. of 3 separated experiments.

To address this, we explored whether echinomycin would overcome the anti-apoptotic impact of Bcl-2 in HT-29 cells by exploiting the controlled Bcl-2 overexpression. This study shows that Bcl-2 regulates echinomycin-induced apoptosis in HT-29 cells.

Echinomycin Treatment Induces Apoptosis of HT-29 Cells

Echinomycin treatment results in apoptosis of many different cell types[9]. To examine the ability of echinomycin to induce apoptosis in HT-29 cells, cultures were treated with a condition of various does and time, thereafter followed by APOPercentage™ apoptosis assay. As shown in Figure 1, echinomycin caused apoptosis of HT-29 cells in a time-dependent manner,



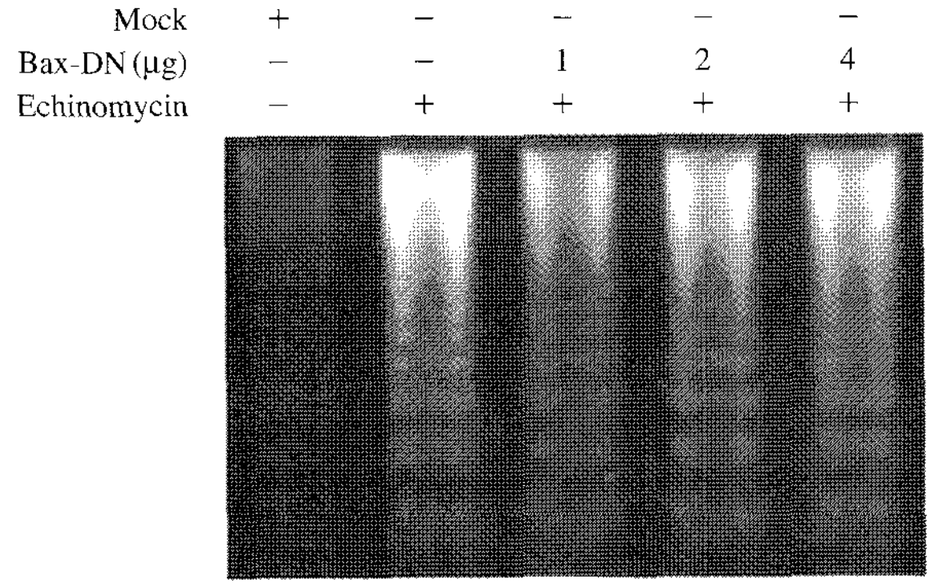


Figure 3. Treanfection of Bax-DN did not protect echinomycin-induced apoptosis. HT-29 cells were transiently transfected with an empty vector as a control or pcDNA3/Bax-DN. Then, 2 μg/mL echinomycin was added to HT-29 cells for 24 h. (A) Bax-DN was determined by immunoblotting in a dose-dependent manner. For immunoblotting, anti-actin antibody was used as a loading control. (B) Apoptotic DNA fragmentation was determined by agarose gel electrophoresis.

from a concentration of 0.2 μg/mL echinomycin. Figure 1 clearly showed that echinomycin drives apoptosis of HT-29 cells.

Echinomycin Induces Mitochondrial Membrane Depolarization

To determine the depolarization of mitochondria in response to echinomycin, the membrane potential-sensitive dye JC-1 was added to cultures of control and echinomycin-treated cells. Thereafter, the fraction of cells with the depolarized mitochondria was quantified by flow cytometry. The mitochondrial depolarization was seen in as early as 6 h (Figure 2). This loss of mitochondrial potential was increased time dependently

Transiently Transfected with Bax-dominant Negative Fails to Protect Echinomycin-induced Apoptosis

The simple correlation between Bcl-2 family expres-

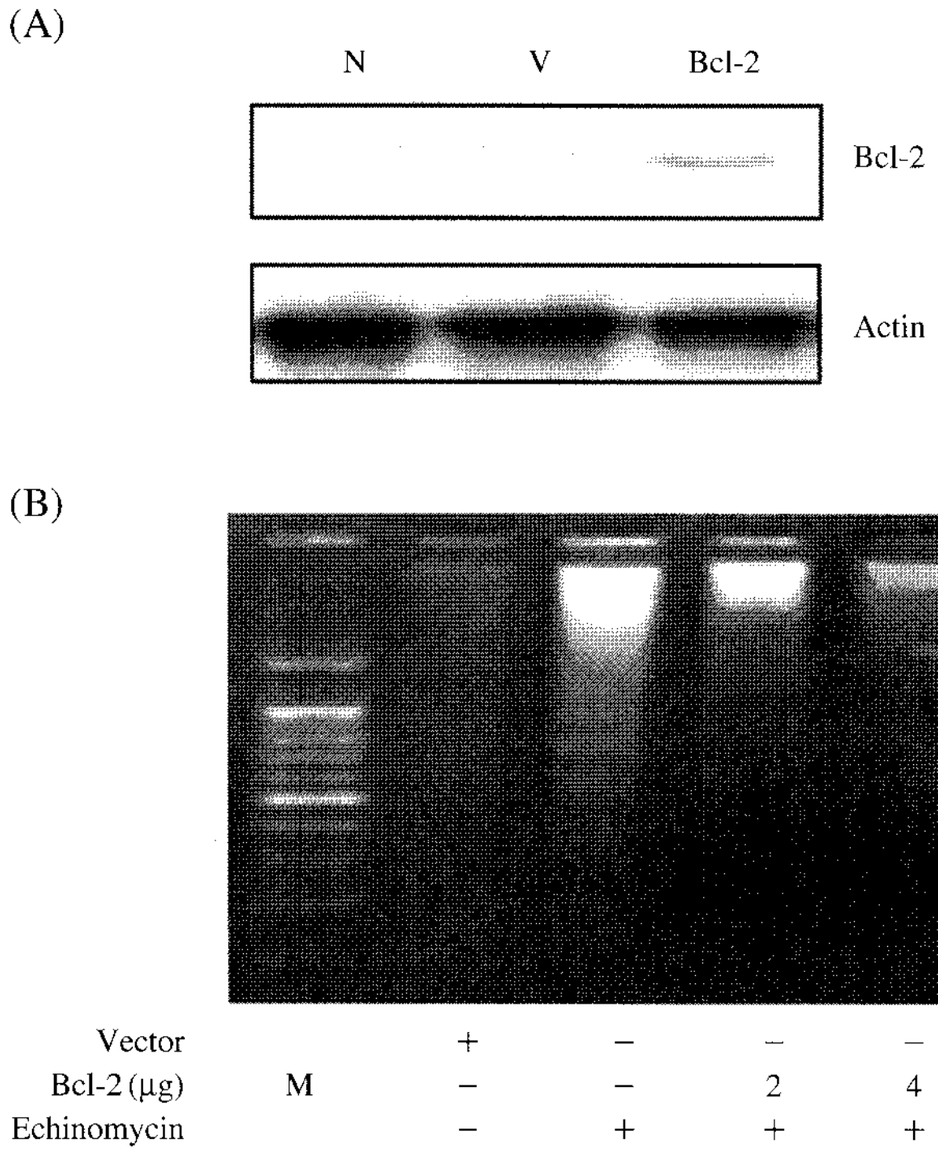


Figure 4. Overexpression of Bcl-2 blocks echinomycin-induced apoptosis. (A) Overexpressed Bcl-2 expression was determined by immunoblotting. Immunoblotting with anti-actin antibody was used as a loading control. HT-29 cells were transiently transfected with an empty vector or pcDNA3 /Bcl-2. Then, 2 μg/mL echinomycin was added to HT-29 cells for 24 h. (B) Apoptotic DNA fragmentation was determined by agarose gel electrophoresis (M: marker). (C) Cells were stained with Annexin V-FITC and propidium iodide (PI). Apoptotic cells are determined by counting the % of Annexin V-FITC (+), PI (−) cells and the % of Annexin V-FITC (+), PI (+) cells. Results shown in figure are expressed as the mean ± S.D of at least 3 separate experiments.

sion and echinomycin-induced apoptosis was examined in previous study[6]. Here, in order to further explore the effect of Bax expression to prevent echinomycin-induced apoptosis in HT-29 cells, HT-29 cells were transiently transfected with Bax-DN prior to echinomycin treatment. Immunoblotting confirmed that the transfectants expressed lowly levels of Bax in echinomycin treatment at 24 h (Figure 3A), and the Bax-DN in HT-29 cells was not changed throughout the apoptosis process after echinomycin treatment (Figure 3B).

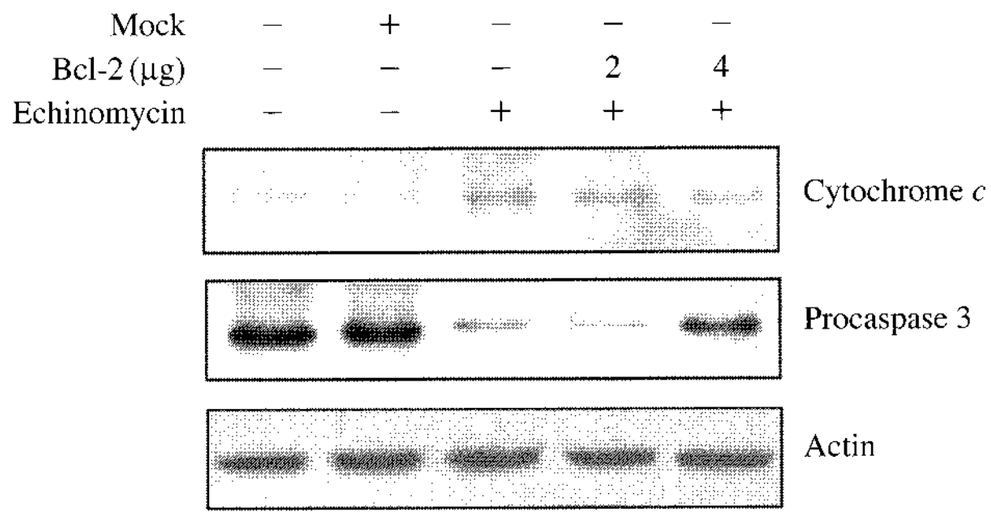


Figure 5. Overexpression of Bcl-2 blocks echinomycin-induced cytochrome *c* release and caspase-3 activation. HT-29 cells were transiently transfected with an empty vector or pcDNA3/Bcl-2. Then, 2 μg/mL echinomycin was added to HT-29 cells for 24 h. Cytochrome *c* and procaspase-3 were detected by immunoblotting with corresponding antibodies. Similar results were achieved in three separate experiments with comparable outcomes. Actin was used as a loading control.

Overexpression of Bcl-2 Inhibits Echinomycin-induced Apoptosis in a Dose-dependent Manner

While mitochondrial dysfunction triggered by echinomycin was partially elucidated, the role of Bcl-2 in this signaling pathway has been still unclear. Thus, HT-29 cells were transiently transfected with Bcl-2 DNA prior to echinomycin treatment. Immunoblotting showed that the transfectants expressed high levels of Bcl-2 (Figure 4A). To further confirm this, HT -29 cells were overexpressed by using Bcl-2 and then treated with echinomycin for 24 h. It is clear that echinomycin-induced apoptosis in HT-29 cells was drastically attenuated by Bcl-2 transfection, whereas a control vector rarely affected it. This clear proof was fully evidenced by DNA fragmentation (Figure 4B) and flow cytometric analysis (Figure 4C). Given this, these data shows that echinomycin-induced apoptosis is indeed sensitive to the overexpression of Bcl-2.

Overexpression of Bcl-2 Inhibits Caspase-3 Activation by Echinomycin Treatment in HT-29 Cells

Echinomycin-induced apoptosis through sequential activation of ERK-casapase pathway was shown in

previous study[6]. But, the relationship between Bcl-2 and caspase activation in echinomycin-induced apoptosis was unknown. To clarify this issue, we examined if Bcl-2 overexpresion would abrogate the cytochrome *c* release as well as the activation of caspase-3. Figure 5 clearly shows that Bcl-2 overexpression abrogates cytochrome *c* release as well as the cleavage of procaspase-3 in a transfected dose-dependent fashion.

Discussion

This study verifies that Bcl-2 regulates echinomycin-induced apoptosis in HT-29 cells. This verifying evidence was that using combined analyses of DNA fragmentation plus flow cytometric analysis, echinomycin-induced apoptosis was drastically attenuated by Bcl-2 overexpression, whereas a control vector rarely affected echinomycin-induced apoptosis. Indirect evidence was that transfection of Bax-donimant negative DNA failed to prevent echinomycin-induced apoptosis.

The Bcl-2 was known to play a pivotal role in promoting tumor cell survival through inhibition of apoptotic cell death following a variety of stimuli[10]. The Bcl-2 family as apoptosis regulators holds anti-(Bcl-2 and Bcl-x_L) and pro-apoptotic (Bax, Bad, Bid, Bik, Bak, and Bcl-x_S) effect[11].

To explore the involvement of Bcl-2 family in echinomycin-mediated apoptosis, preliminary experiment using immunoblotting has shown that the down-regulation of anti-apoptotic protein Bcl-2 as well as the up-regulation of pro-apoptotic protein Bax could trigger mitochondria-mediated apoptosis on HT-29 cells[6]. This clue provides two plausible hypotheses: 1) Bcl-2 dependent, not Bad-dependent or 2) Bcl-2 independent, not Bad-dependent. However, previous experiment had some defects in terms of failure to prove the regulatory machinery between Bcl-2 and Bad at upstream level of mitochondria-MAPKs-capsase pathway in echinomycin-mediated apoptosis. To resolve this, functional opposite expression system: 1) Bcl-2 overexpresson 2) transfection of Bax-DN. Major hypothesis was that Bcl-2 might regulate echinomycin-mediated apoptosis in HT-29 cells. In initial experiment to rule out the possible engagement of Bax, it was found that overexpression of Bax-DN failed to block echinomycin-induced apoptosis in HT-29 cells (Figure 3), indicating that echinomycin-induced apoptosis of HT-29 cells may be independent of Bax expression. In this care, data coupled with previous data (down-regulation of anti-apoptotic protein Bcl-2) indirectly verify this study hypothetical path (Bcl-2 dependent, not Bad-dependent). It was well documented that Bcl-2 overexpression protects apoptosis induced by anticancer agents[12,13]. In particular, DNA-damaging agent was known to cause apoptosis via direct or indirect inhibition of Bcl-2 expression[14]. However, this research was the first suggestion that of diverse, structured minor groove binders (MGB), the prototypic echinomycin might control the apoptotic signaling via Bcl-2-mitochondria pathway.

Previous study showed that echinomycin-induced apoptosis through sequential activation of ERK-caspase pathway[6]. But, the direct linkage between Bcl-2 and caspase activation in echinomycin-induced apoptosis was elusive. On this question, these data (Figure 5) clearly indicatedthat Bcl-2 overexpression abrogates cytochrome *c* release as well as the activation of procaspase-3 in a transfected dose-dependent fashion. These results were not clearly compatible with the pattern of apoptosis triggered by DNA damaging or intercalating agents[15,16].

Several line of evidences suggested that mitochondrial damage might cause degradation of Bcl-2[8,17]. Consistently, we demonstrated that caspase-3 and cytochrome *c* release were directly involved in the execution of echinomycin-induced apoptosis in HT-29[6]. It was generally accepted that the cytochrome *c* release and depolarization are essential to mitochondrial apoptosis[18]. Given this, echinomycin triggers the depolarization of mitochondria in the presence of cytochrome *c* release, followed by the activation of caspase-3. Current data plus a previous report clearly reconfirm that the mitochondrial dysfunction was requisite to echinomycin-mediated apoptosis.

These cumulative results clearly indicated that echinomycin-mediated apoptosis of HT-29 cells occurs via Bcl-2-mitochondria path. Another important implication was to unravel the hidden apoptotic signal path regarding MGB or DNA bis-intercalating agent. Collectively, these data verify that Bcl-2 regulates echinomycin induced apoptosis in HT-29 cells. This discovery will not suffice to uncover the full scenario of echinomycin-induced apoptosis. Every MGB might take a unique signal path. In that context, further discovery of a novel signal path taken by echinomycin or other MGB should shed light on the creation of novel therapeutics against aberrant signal-transducing disease, such as cancer, hyper-proliferative disease, infections.

Methods

Chemotherapeutics

Echinomycin (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St.

Louis, MO, USA) and added to the culture medium at the indicated concentration. The concentration of DMSO in the medium was less than 1% (v/v). Cells were incubated 37°C for the indicated times and harvested.

Cell Culture

Human colon cancer cell line HT-29 was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). HT-29 cells were cultured in RMPI1640 (Gibco BRL, Hercules, CA, USA) and supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 U/mL) in an atmosphere of 5% CO_2 in air at 37°C.

Preparation of Cell Extracts

Cells from a dish were harvested, pelleted, and washed in phosphate-buffered-saline (PBS). The cell pellet was then resuspended in an equal volume of lysis buffer (100 mM Tris, 150 mM NaCl, 10% glycerol, 0.6% Triton-X 100, 5 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were incubated for 60 min on ice, and centrifuged at 14,000 rpm for 30 min at 4°C. The soluble fraction was transferred to a new tube, and the preparation was stored at −70°C.

DNA Fragmentation Analysis

Approximately 2×10^6 cells were lysed with 0.2 mL of lysis buffer containing 10 mM Tris-HCl, 20 mM EDTA, and 0.5% Triton X-100 (pH 8.0), and placed on ice for 30 min. Cell extracts were clarified by centrifugation at 14,000 rpm for 10 min. The supernatant containing DNA cleavage products was precipitated overnight using isopropyl alcohol (Merck, Clarkston, MI, USA). The lysates was incubated with 0.3 mg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) at 37°C for 1 h. Dry DNA pellets were then resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA), containing 0.5 mg/mL RNase A (Boehringer Mannheim, Manneheim, Germany). DNA fragments were separated on a 1.8% agarose gel, and visualized by ethidium bromide staining (Bio-Rad, Hercules, CA, USA), and photographed.

Apoptosis Assay

HT-29 cells were seeded in 35-mm plastic dishes (3×10^5 cells per dish) and treated with echinomycin for 24 h. Cells were prepared and analyzed using APOPercentage™ apoptosis assay according to the manufacturer's instructions (Biocolor, Newtonabbey, Northern Ireland).

Quantitative Analysis of Apoptosis by Flow Cytometry

Cells were harvested, washed with PBS, and resuspended in a binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After 15 min of incubation with Annexin V-fluorescein isothiocyanate (Sigma, St. Louis, MO, USA) and propidium iodide (PI) (Pharmingen, San Diego, CA, USA) at room temperature, the fluorescence emitted by cells (10,000 cells/sample) was analyzed on a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western Blot Analysis

HT-29 cells were seeded in 35-mm plastic dishes (3×10^5 cells per dish) and incubated with echinomycin for different time periods. Cells were lysed in the lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1 mM sodium orthovanadate). After centrifugation at 15,000 rpm at 4°C for 30 min, supernatant was collected, 20 μg of lysates from each sample was run on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were rinsed in TBST (10 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.05% Tween 20, and 1 mM EDTA) and blocked in blocking buffer (TBST containing 5% bovine serum albumin) overnight at 4°C. PVDF membranes were incubated with primary antibodies overnight at 4°C, washed, and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) or goat anti-mouse IgG conjugated with HRP for 1 h at room temperature. The membrane was developed with electrogenerated chemiluminescent (ECL) substrate (Amersham Life Sciences, Arlington Heights, IL, USA), and exposed to Biomax MS autoradiography x-ray film (Kodak, Rochester, NY, USA).

Detection of Cytochrome *c* Release

1×10^7 cells were trypsinized and collected at the indicated times. The pellets were washed with ice-cold PBS and resuspended in 150 μL of cytosol extraction buffer (250 mM sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). The cell pellets were then homogenized with 30 strokes of a homogenizer (Wheaton, Millville, NJ, USA). The mitochondria-enriched fraction was pelleted by centrifugation at 14,000 rpm for 30 min. The supernatant was subjected to Western blot analysis with a monoclonal antibody to cytochrome *c*.

Transfection

Transfection was performed using LipofectAMINE-2000 (Invitrogen, Carlsbad, CA, USA), according to the supplier's protocol. HT-29 cells were plated in 6-well plates (1×10^6 cells/well) and transfected with each expression vector (pcDNA3-Bcl-2, or pcDNA3-Bax-dominalt negative (DN)) or with a control vector (pcDNA3, empty vector). The plasmids used in this experiment were kindly provided by Dr. Kim, H. S. (University of Catholic, Republic of Korea). Twenty-four hours after transfection, the cells were treated with echinomycin for different time periods as indicated for Western blot analysis, DNA fragmentation and quantitative analysis of apoptosis by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

Mitochondrial Depolarization

To measure mitochondrial depolarization, drug-treated or untreated cells (5×10^6 cells/mL) were incubated with 5 μg/mL JC-1 (5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazoly-carbocyanine iodide) as described in HT-29 cells. After incubation for 20 min at room temperature in the dark, cells were washed once with PBS. Flow cytometry was performed on a FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical Evaluation

All experiments were performed at least three times. Results are presented as means ± standard deviation (SD) if not otherwise indicated. Significance of the results was analyzed by the Student's *t*-test.

Acknowledgements

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