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Molecular mechanisms of ZD1839 (Iressa)-induced apoptosis in human leukemic U937 cells[1]

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Key words

ZD1839 (Iressa); apoptosis; caspase-3; Bcl-2; mitogen-activated protein kinases

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Abstract

Aim: To investigate the molecular mechanisms of ZD1839-induced apoptosis in human leukemic U937 cells. **Methods:** The inhibition of human leukemic U937 cell growth was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2*H*-tetrazolim bromide (MTT) assays, lactate dehydrogenase (LDH) release, and cell cycle distribution. The expression of anti- and pro-apoptotic proteins was detected by Western blot analysis. **Results:** This study demonstrated that ZD1839 induced apoptosis in leukemic U937 cells by the downregulation of Bcl-2, caspase activation and subsequent apoptotic features. Cotreatment with ZD1839 and the caspase-3 inhibitor z-DEVD-fmk blocked apoptosis, indicating that caspase-3 activation is at least partially responsible for ZD1839-induced apoptosis. The ectopic expression of Bcl-2 attenuated caspase-3 activation, PARP cleavage, and subsequent indicators of apoptosis, including sub-G_1 DNA content and LDH release. These results indicate that the downregulation of Bcl-2 plays a major role in the initiation of ZD1839-induced apoptosis, and that the activation of a caspase cascade is involved in the execution of apoptosis. Furthermore, ZD1839 treatment triggered the activation of p38 mitogen-activated protein kinase (MAPK) and the downregulation of c-Jun-N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and phosphatidyl inositol 3-kinase (PI3K)/Akt. The inhibition of the ERK and PI3K/Akt pathways also significantly increased cellular death. **Conclusion:** ZD1839 activated caspase-3 and the inhibited Bcl-2 in human leukemic U937 cells through the downregulation of the ERK and PI3K/Akt pathways.

Introduction

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase involved in multiple physiological processes including cell proliferation, survival and migration[1]. EGRF is highly expressed in solid tumor cells such as the breast, lung, colon, ovaries and brain[2]. According to previous studies, the constitutive expression of EGFR activates intracellular signal transduction cascades involving the Ras-Raf-mitogen-activated protein kinase (MAPK), phosphatidyl inositol 3-kinase (PI3K)/Akt, and signal transducer and activator of transcription (STAT) pathways[3]. All of these pathways have been implicated in the inhibition of apoptosis and the promotion of tumor cell motility[4,5]. Therefore, drugs and monoclonal antibodies designed to inhibit EGFR tyrosine kinase activity are actively being developed for cancer therapy[6–8].

ZD1839 (Iressa) is a quinazoline derivative that inhibits EGFR tyrosine kinase activity by binding to the adenosine triphosphate pocket within EGFR's catalytic domain[9]. Although ZD1839 has been proposed as a biologically targeted agent for the treatment of cancer[10–14], Govindan *et al*[15] proved that ZD1839 was not active in malignant mesothelioma, regardless of EGFR expression. The clustered incidence

of acute promyelocytic leukemia was also detected during ZD1839 treatment of non-small-cell lung cancer[16]. On the other hand, Stegmaier *et al*[17] recently reported that ZD1839 induced myeloid differentiation of acute myeloid leukemia without EGFR at clinically achievable doses. However, the mechanisms responsible for its effects on leukemia cells without EGFR have yet to be fully elucidated.

In the present study we examined the effect of ZD1839 on antiproliferation and apoptosis in human leukemic U937 cells without EGFR. The results provide new insight about the function of Bcl-2 and caspase-3 during apoptosis in response to ZD1839 exposure in leukemic U937 cells. Our results also demonstrate that ZD1839 can initiate a substantial apoptotic response through the downregulation of extracellular signal-regulated kinase (ERK) and PI3K/Akt.

Materials and methods

Reagents Propidium iodide (PI), 4,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphnyl-2*H*-tetrazolim bromide (MTT) were purchased from Sigma (St Louis, MO, USA). Caspase activity assay kits were purchased from R&D systems (Minneapolis, MN, USA). An enhanced chemiluminescence (ECL) kit was purchased from Amersham (Arlington Heights, IL, USA). Caspase-3 inhibitor 1 z-DEVD-fmk, PD98059, SP600125, SB203580, and LY294002 were obtained from Calbiochem (San Diego, CA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen Co (Carlsbad, CA, USA) and GIBCO-BRL (Gaithersburg, MD, USA), respectively. All other chemicals not specifically cited here were purchased from Sigma (USA).

Antibodies Anti-inhibitor of apoptosis protein (cIAP)-1, anti-cIAP-2, anti-X-linked inhibitor of apoptosis (XIAP), anti-Bcl-2, anti-Bax, anti-Bad, anti-poly (ADP-ribose) polymerase (PARP), anti-phospholipase (PLC)-γ1, anti-caspase-3, anti-caspase-8, anti-caspase-9, antibodies against PI3K and phosphor (p)-PI3K, respectively, were purchased form Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ERK, p-ERK, p38, p-p38, c-Jun-N-terminal kinase (JNK), p-JNK, Akt and p-Akt were purchased from PharMingen (San Diego, CA, USA), and the antibody against β-actin was from Sigma (USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham (USA).

Cell culture Human leukemic U937 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Bcl-2-overexpressing U937 (U937/Bcl-2) cells were kindly provided by Professor TK KWON (Department of Immunology, School of Medicine, Keimyung University, Daegu, South Korea) in South Korea. The cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and maintained in RPMI-1640 medium containing 10% heat-inactivated FBS.

Cell viability and proliferation The cells were treated with the indicated concentrations of ZD1839 for 48 h, and the cell number and viability were determined by trypan blue exclusion staining and MTT assays, respectively. The control cells were supplemented with complete media containing 0.1% DMSO as a vehicle control for 48 h.

Nuclear staining After treatment with ZD1839, the cells were harvested, washed in ice-cold phosphate-buffered solution (PBS), fixed with 3.7% paraformaldehyde, and then permeabilized in PBS containing Triton X-100. The fixed cells were washed with PBS and the nuclei were stained with DAPI solution. Nuclear morphology was evaluated by fluorescence microscopy.

Cell cycle analysis The cells were serum-starved for 24 h in order to synchronize them in the G_0 phase of the cell cycle. Synchronous populations of cells were treated with ZD1839 for 48 h. The cells were fixed in 75% (*v*/*v*) ethanol for 1 h at 4 °C and resuspended in cold PI solution (50 μg/mL) containing RNase A (0.1 mg/mL) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry was performed using FACSCalibur (Becton Dickinson, San Jose, CA, USA). Forward light scatter characteristics were used to exclude the cell debris from the analysis. The sub-G_1 population was calculated in order to estimate the apoptotic cell population.

Protein extraction and Western blot analysis The cells were harvested and gently lysed for 2 min in ice-cold lysis buffer [20 mmol/L sucrose, 1 mmol/L EDTA, 20 μmol/L Tris-Cl (pH 7.2), 1 mmol/L dithiothreitol, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 5 μg/mL pepstatin A, 10 μg/mL leupeptin and 2 μg/mL aprotinin]. The lysates were centrifuged at 14 000×*g* at 4 °C for 10 min. The supernatant was collected and the protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were either stored at -80 °C or used immediately for the Western blot analysis. Aliquots containing 30 μg total protein were separated on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The proteins were detected using an ECL detection system (Amersham, USA).

Determination of caspase activity Caspase activities were determined by a colorimetric assay according to the manufacturer's instructions. Briefly, the cells were lysed in the supplied lysis buffer. The supernatant was collected and then incubated with the supplied reaction buffer containing

dithiothreitol and substrate. The reaction was measured by changes in absorbance at 405 nm using the VERSA-max microplate reader (Molecular Devices, Palo Alto, CA, USA).

DNA fragmentation assay U937 and U937/Bcl-2 cells were treated with different concentrations of ZD1839 for 48 h and were lysed on ice in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, and 0.5% Triton X-100 for 30 min. The lysates were vortexed and cleared by centrifugation at 10 000×*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol (25:24:1, *v/v/v*) and analyzed electrophoretically on 1.5% agarose gel containing ethidium bromide.

Determination of cytotoxicity For the determination of plasma membrane integrity loss, lactate dehydrogenase (LDH) release into the extracellular medium was measured using the cyto-tox96 non-radioactive assay from Promega (Madison, WI, USA) in order to determine cytotoxicity. This assay measures the formation of a red formazan product after the conversion of lactate and nicotinamide adenine dinucleotide (NAD^+) to pyruvate and NAD^+ hydrogen. The assay was used according to the manufacturer's instructions. Briefly, the maximum release of LDH was obtained by adding 100 μL of 2% Triton X-100 to the untreated cells. One hundred microliters of each sample were incubated with 100 μL of LDH assay reagents for 10 min and the absorbance of the samples was measured at 490 nm. The percentage of LDH release was determined by dividing the amount of LDH released by the cells under each condition by the maximum amount of LDH release, and then multiplying the fraction by 100.

Statistical analysis All data are presented as mean±SD. Significant differences between the groups were determined using the unpaired Student's *t*-test. A value of $P<0.05$ was accepted as an indication of statistical significance. All the figures shown were obtained from at least 4 independent experiments with a similar pattern.

Results

ZD1839-induced apoptosis in a concentration-dependent manner in U937 cells We first evaluated the ability of ZD1839 to attenuate the proliferation and viability of U937 cells by cell-counting and MTT assay. As shown in Figure 1A and 1B, ZD1839 treatment significantly decreased cell proliferation and viability in a concentration-dependent manner. Cellular death was not observed when treated with ZD1839 at a concentration of 10 μmol/L, however, a marked decrease in cell numbers [(465±21)×10^3 cells/mL)] and viability (69%±4%) was observed at 15 μLmol/L. Exposure to 25 μmol/L of ZD1839 sharply decreased cell numbers and viability to (332±35)×10^3 cells/mL and 46%±7%, respectively. Additionally, the U937 cells showed a marked change in morphology including shrinkage, irregular shape, and condensed chromatin in their nuclei (Figure 1C) following 48 h of exposure to 20 or 25 μmol/L ZD1839. The cell cycle analysis also revealed that 25 μmol/L ZD1839 significantly increased the sub-G_1 DNA content (33%±4%), indicating apoptosis (Figure 1D). These data indicate that ZD1839 inhibits cell proliferation and induces cell death in U937 cells.

Caspase-3 is a potential target of ZD1839-induced apoptosis In order to examine whether caspases were likely involved in the apoptotic response, the expression and the activation of caspases-3, -8, and -9 were evaluated following treatment with ZD1839 (Figure 1). The cells were treated with ZD1839 at the indicated concentrations for 48 h, and the caspase activity from the cell lysates was determined. As shown in Figure 2A, ZD1839 triggered caspase-3 activation in a dose-dependent manner; however caspases-8 and -9 were activated only at ZD1839 concentrations greater than 20 μmol/L. The activation of caspases was also confirmed by the decrease of proform caspase by Western blot analysis (Figure 2B). In a parallel experiment, treatment with ZD1839 at concentrations of more than 20 μmol/L decreased procaspases-3, -8, and -9, followed by increases in PARP cleavage and PLC-γ degradation. We next attempted to determine whether caspase-3 was likely to play an important role in ZD1839-induced apoptosis by treating cells with the specific caspase-3 inhibitor z-DEVD-fmk. The inhibition of caspase-3 activity by pretreatment with 50 μmol/L z-DEVD-fmk significantly decreased the appearance of sub-G_1 DNA content and apoptotic bodies following ZD1839 treatment (Figure 2C). Additionally, the DNA fragmentation (Figure 2D) and LDH release (Figure 2E) associated with ZD1839 were significantly attenuated 1 h after pretreatment with z-DEVD-fmk. Furthermore, ZD1839 treatment was associated with the appearance of caspase-3 and PARP cleavage products; however, a significant decrease in these products was observed following pretreatment with z-DEVD-fmk (Figure 2F). These results suggest that ZD1839-induced apoptosis may be executed by activating the caspase-3 pathway.

Ectopic expression of Bcl-2 significantly attenuates ZD1839-induced apoptosis Bcl-2 and IAP family members could ultimately inhibit or promote apoptosis. Therefore, we also investigated whether ZD1839 treatment can modulate the expression of these pro- and anti-apoptotic proteins. Western blot analysis revealed that ZD1839 significantly

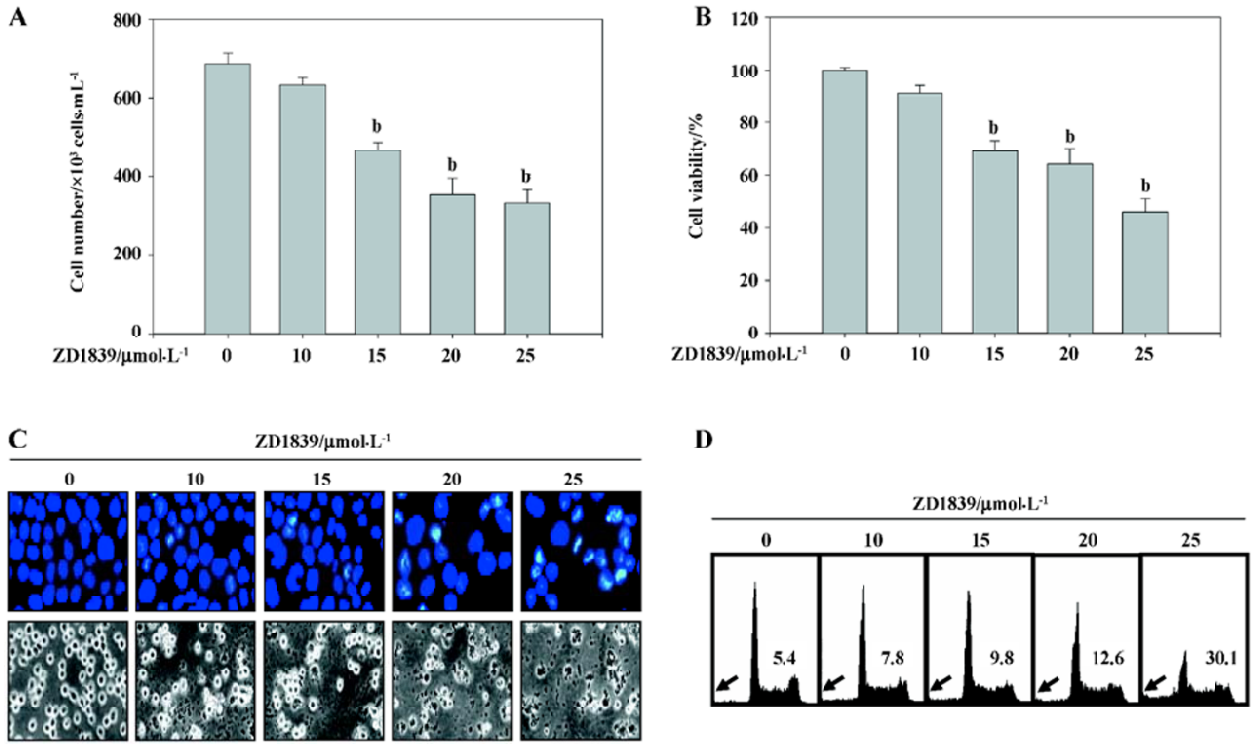

Figure 1. ZD1839 inhibits proliferation and induces cell death in U937 cells. Cells were seeded at a density of 2×10^4 cells/mL and treated with the indicated concentrations of ZD1839 for 48 h. (A) Cell numbers and (B) Viability were determined by hemocytometer counts of trypan blue-excluding cells and MTT assays, respectively. (C) Nuclear (upper panels) and cellular (lower panels) morphology of cells incubated with or without ZD1839 for 48 h was examined under fluorescence and light microscopy (×400), respectively. (D) DNA content was analyzed by flow cytometry. Arrows indicate the population of cells in the G_1 phase, as analyzed by CellQuest software. DMSO (0.1%), used as a vehicle control did not affect cell proliferation, viability, or morphology (data not shown). One representative experiment of 3 performed results that showed similar patterns. Significance was determined by a Student's *t*-test ($^bP<0.05$ *vs* untreated control).

downregulated anti-apoptotic proteins such as Bcl-2, cIAP-1 and XIAP, and upregulated the pro-apoptotic protein Bax in a dose-dependent manner (Figure 3A). However, ZD1839 had no effect on the levels of Bad or cIAP-2. These results indicate that ZD1839 is likely to induce apoptosis in U937 cells by downregulating the expression levels of Bcl-2, cIAP-1, XIAP, and upregulating Bax. In order to further elucidate the role of Bcl-2 in ZD1839-induced apoptosis in U937 cells, we next investigated the suggestive apoptotic features in U937/Bcl-2 cells. Western blot analysis confirmed that U937/Bcl-2 cells had significantly higher levels of Bcl-2 than the control U937 cells (data not shown). The ectopic expression of Bcl-2 also completely protected U937 cells from the formation of sub-G_1 and apoptotic bodies induced by ZD1839 (Figure 3B). Although ZD1839 clearly induced a potent DNA fragmentation and LDH release in U937 cells after 48 h of treatment, these features were not observed or were significantly reduced in U937/Bcl-2 cells (Figures 3C, D). Consistent with the inhibition of apoptosis, the cleavage of caspase-3 and PARP in U937/Bcl-2 cells was significantly reduced. These results indicate that the downregulation of Bcl-2 proteins might be associated with ZD1839-induced apoptosis in U937 cells, and might act as an upstream regulator of caspase-3 and PARP.

Inhibition of the ERK pathway increases ZD1839-induced apoptosis In order to investigate the role of the MAPK signaling pathway in ZD1839-induced apoptosis, we investigated the effect of ZD1839 on the expression and function of MAPK. As shown in Figure 4A, the phosphorylation of p38 MAPK increased significantly from 24 to 48 h after ZD1839 treatment. However, ERK and JNK activation gradually decreased after 24 h of ZD1839 treatment. We next evaluated the possible roles of MAPK in ZD1839-induced apoptosis. As shown in Figures 4B and 4C, treatment with SB203580, a specific p38 MAPK inhibitor, slightly increased cellular viability (55%±4%), although this increase was not statistically significant. However, pretreating cells with PD98059, a potent inhibitor of ERK, modestly increased

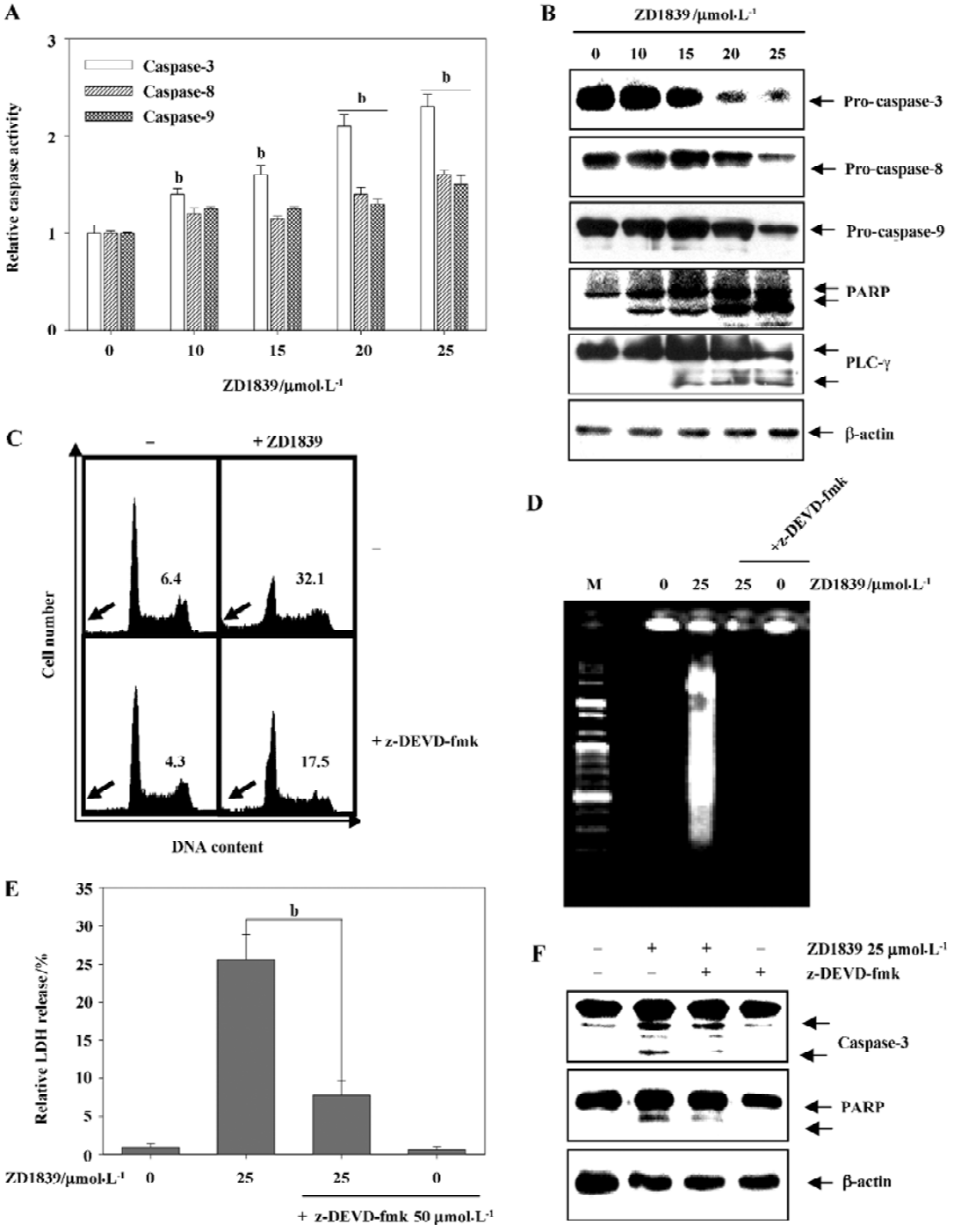

Figure 2. Caspase-3 inhibitor z-DEVD-fmk alleviates ZD1839-induced apoptosis in U937 cells. U937 cells were treated with the indicated concentration of ZD1839 for 48 h. (A) Caspase activities were determined following the manufacturer's protocol. (B, F) Total cell lysates were prepared and aliquots containing 30 μg of protein were subjected to SDS–PAGE followed by Western blot analysis with specific antibodies. (C) U937 cells were incubated with 25 μmol/L ZD1839 for 48 h after 1 h pretreatment with z-DEVD-fmk (50 μmol/L). Cells were analyzed by flow cytometry. Arrows indicate the population of cells with sub-G_1 phase DNA content, suggesting apoptosis. (D) Fragmented DNA was extracted and analyzed on 1.5% agarose gel containing ethidium bromide. (E) LDH release was quantified as a marker of cytotoxicity in the presence of z-DEVD-fmk. Results are from 1 representative experiment of 3 performed results that show similar patterns. Each point represents the mean±SD of 3 independent experiments. Significance was determined by a Student's *t*-test ($^bP<0.05$ *vs* untreated control).

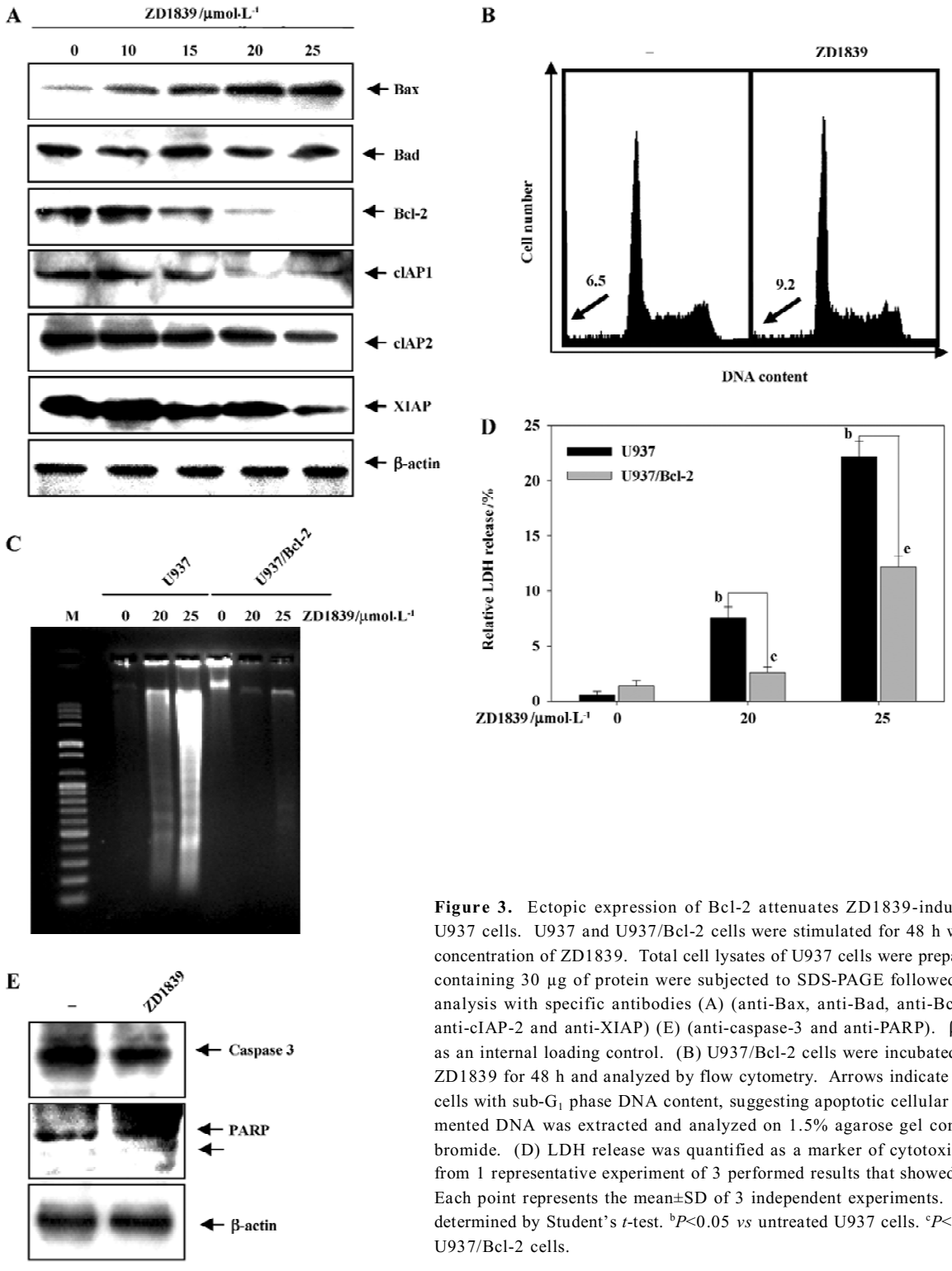

Figure 3. Ectopic expression of Bcl-2 attenuates ZD1839-induced apoptosis in U937 cells. U937 and U937/Bcl-2 cells were stimulated for 48 h with the indicated concentration of ZD1839. Total cell lysates of U937 cells were prepared, and aliquots containing 30 μg of protein were subjected to SDS-PAGE followed by Western blot analysis with specific antibodies (A) (anti-Bax, anti-Bad, anti-Bcl-2, anti-cIAP-1, anti-cIAP-2 and anti-XIAP) (E) (anti-caspase-3 and anti-PARP). β-Actin was used as an internal loading control. (B) U937/Bcl-2 cells were incubated with 25 μmol/L ZD1839 for 48 h and analyzed by flow cytometry. Arrows indicate the population of cells with sub-G_1 phase DNA content, suggesting apoptotic cellular death. (C) Fragmented DNA was extracted and analyzed on 1.5% agarose gel containing ethidium bromide. (D) LDH release was quantified as a marker of cytotoxicity. Results are from 1 representative experiment of 3 performed results that showed similar patterns. Each point represents the mean±SD of 3 independent experiments. Significance was determined by Student's *t*-test. $^bP<0.05$ *vs* untreated U937 cells. $^cP<0.05$ *vs* untreated U937/Bcl-2 cells.

the sub-G_1 phases (32%±4%) and decreased cellular viability (35%±6%) in the presence of ZD1839. Interestingly, treating U937 cells with 5 μmol/L of SP600125, a potent inhibitor of JNK, reduced the number of cells with sub-G_1 DNA content from 24%±4% to 10%±3% and increased cellular viability from 47%±5% to 92%±4%, but increased arrest to the G_2

phase. Treatment with 5 or 10 μmol/L SP600125 could significantly attenuate the appearance of ZD1839-induced DNA fragmentation and LDH release through an increase in G_2 levels, however, a high dose (20 μmol/L) of SP600125 did not inhibit the appearance of apoptotic features (Figures 4D, E). It is possible that SP600125 itself increased apoptosis or G_2 arrest. These results led us to believe that ZD1839 is likely to downregulate the ERK pathway, suggesting that this pathway is likely involved in ZD1839-induced apoptosis.

PI3K inhibitor LY294002 sensitizes ZD1839-induced apoptosis We further questioned whether ZD1839 alters PI3K and Akt activation in U937 cells. In order to investigate the activation of these cascades, we determine the expression and phosphorylation levels of PI3K and Akt after treatment with 25 μmol/L of ZD1839. The levels of phosphorylated PI3K decreased significantly in response to ZD1839 after treatment for 6 h. Consistent with this, the levels of phosphorylated Akt also decreased from 24 to 48 h (Figure 5A).

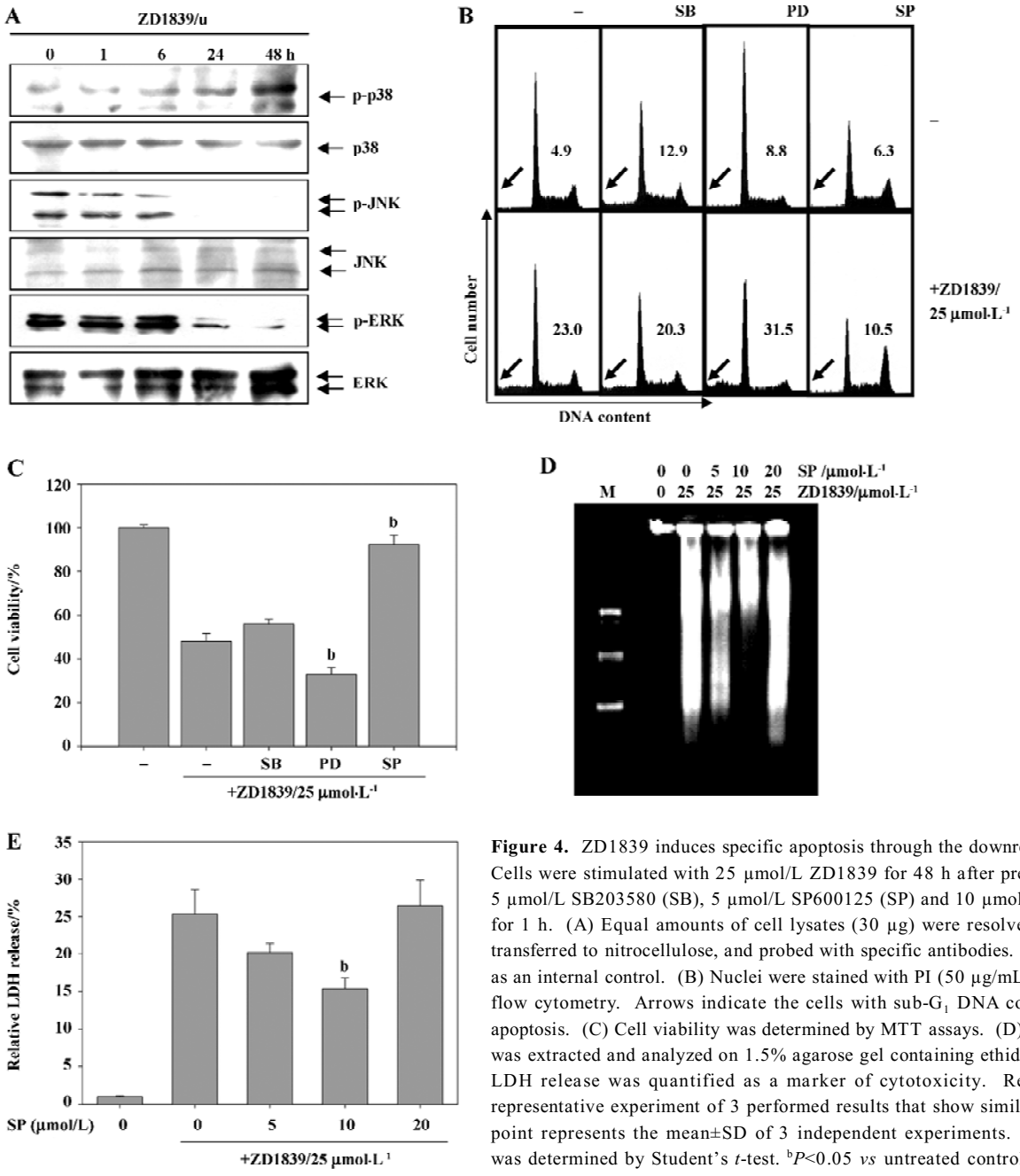

Figure 4. ZD1839 induces specific apoptosis through the downregulation of ERK. Cells were stimulated with 25 μmol/L ZD1839 for 48 h after pretreatment with 2.5 μmol/L SB203580 (SB), 5 μmol/L SP600125 (SP) and 10 μmol/L PD98059 (PD) for 1 h. (A) Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies. β-Actin was used as an internal control. (B) Nuclei were stained with PI (50 μg/mL) and analyzed by flow cytometry. Arrows indicate the cells with sub-G_1 DNA content, suggesting apoptosis. (C) Cell viability was determined by MTT assays. (D) fragmented DNA was extracted and analyzed on 1.5% agarose gel containing ethidium bromide. (E) LDH release was quantified as a marker of cytotoxicity. Results are from 1 representative experiment of 3 performed results that show similar patterns. Each point represents the mean±SD of 3 independent experiments. The significance was determined by Student's *t*-test. $^bP<0.05$ *vs* untreated control.

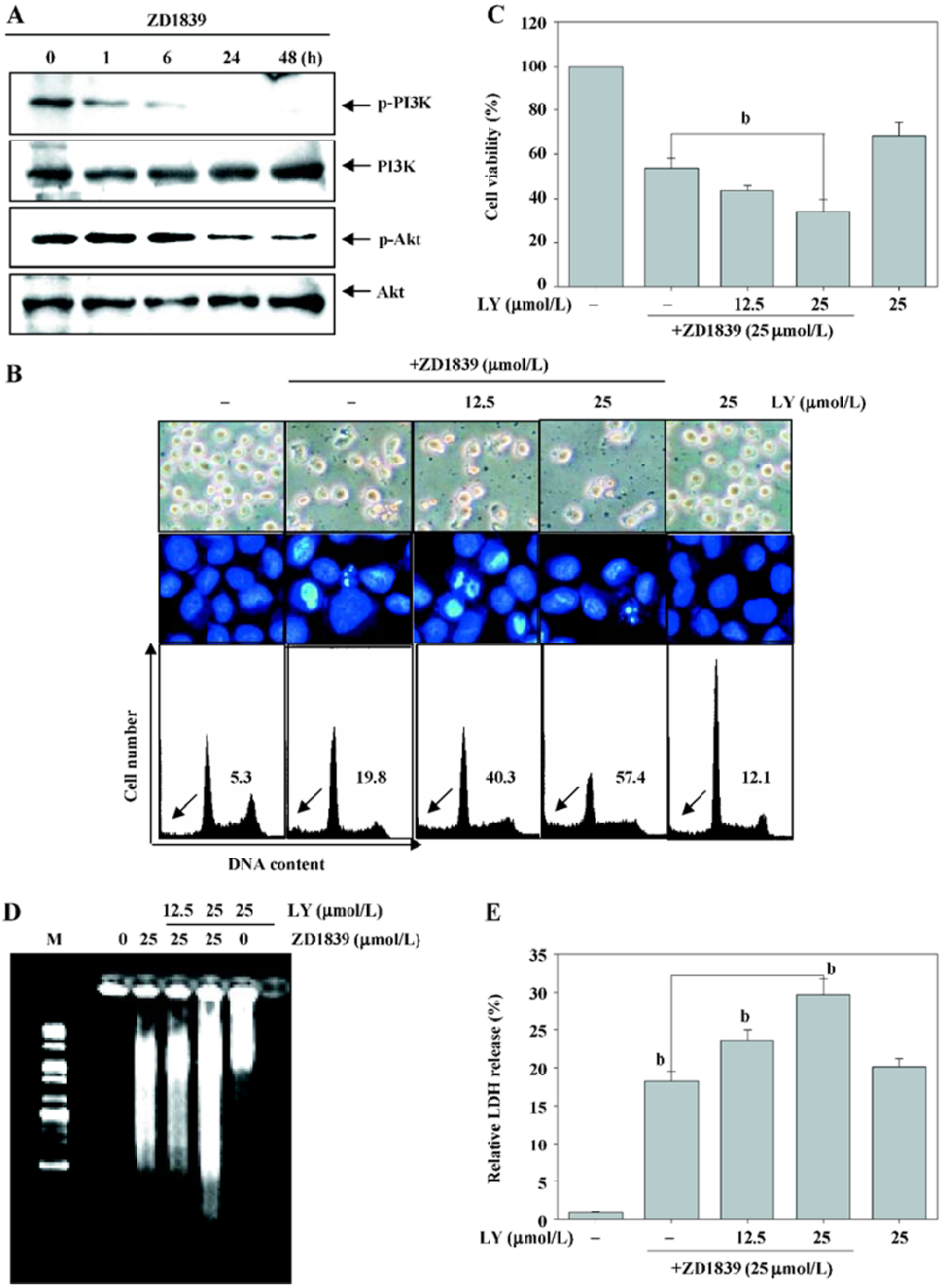

Figure 5. LY294002 (LY) sensitizes ZD1839-induced apoptosis in U937 cells. Cells were treated with 25 μmol/L ZD1839 for 48 h in the absence or presence of 12.5 or 25 μmol/L LY294002. (A) Equal amounts of cell lysate (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies (anti-p-PI3K, anti-PI3K, anti-p-Akt, and Akt, respectively). β-Actin was used as an internal loading control. (B) Cellular morphology was determined (upper panels), and the cells were stained with DAPI (middle panels) and analyzed by flow cytometry (lower panels). Arrows indicate the population of cells with sub-G_1 phase DNA content, suggesting apoptosis. (C) Cell viability was determined by MTT assays. (D) Fragmented DNA was extracted and analyzed on 1.5% agarose gel containing ethidium bromide. (E) LDH release was quantified as a marker of cytotoxicity. Results are from 1 representative experiment of 3 performed results that show similar patterns. Each point represents the mean±SD of 3 independent experiments. Significance was determined by Student's *t*-test. $^bP<0.05$ *vs* untreated control.

The total PI3K and Akt protein levels remained constant at various times points during ZD1839 treatment. We further investigated whether the activation of the PI3K/Akt pathways is necessary for ZD1839-induced apoptosis. The PI3K inhibitor LY294002 was used to determine whether the Akt phosphorylation inhibitor was responsible for the induction of apoptosis. As shown in Figure 5B, the inhibitor (25 μmol/L) alone modestly increased in apoptosis and G_1 arrest, and cotreatment with ZD1839 markedly increased apoptosis, as determined by morphological change, DNA condensation, and sub-G_1 DNA content. We next analyzed cellular viability, DNA fragmentation, and LDH release in order to further elucidate the relationship between the Akt pathway and ZD1839-induced apoptosis in U937 cells. Treatment with 25 μmol/L of LY294002 significantly decreased ZD1839-induced cell viability from 53%±4% to 36%±7% (Figure 5C). We also found that co-treatment with LY294002 and ZD1839 markedly increased the level of DNA fragmentation (Figure 5D) and LDH release (Figure 5E). These results indicate that ZD1839-induced apoptosis may be associated with the downregulation of the Akt signal pathway in this system.

Discussion

In the present study, we first demonstrated that ZD1839 significantly inhibited the proliferation of, and promoted apoptosis in, human leukemic U937 cells without EGFR. Thus, this study indicates that ZD1839-induced apoptosis in U937 cells is mediated by Bcl-2 downregulation, which subsequently activates caspase-3 followed by the cleavage of PARP and PLC-γ for apoptosis. These pathways occurred from unknown causes, because most leukemic cells have little EGFR and are insensitive to low doses.

Bcl-2 and IAP proteins have been investigated as potential therapeutic targets on the basis of their ability to disrupt apoptosis and to confer resistance to chemotherapy in cancer cells[18,19]. Bcl-2 can form ion channels in biological membranes[20], and those channels may control apoptosis by influencing intracellular membranes' permeability and cytochrome c release from mitochondria[21]. In this study, ectopic expression of Bcl-2 and inhibition of caspase-3 significantly promoted cell viability. In contrast, Chang *et al*[22] found that ZD1839 did not change Bcl-2 or Bax expression, and that ectopic expression of Bcl-2 could not prevent ZD1839-induced apoptosis, thus suggesting that a Bcl-2 protein-independent pathway was associated with ZD1839-mediated apoptosis in human lung adenocarcinoma A549 cells. Conversely, some researchers have reported that ZD1839-induced apoptosis involves a Bcl-2-dependent mechanism[11] and that combined targeted inhibition of Bcl-2 and other anti-apoptotic proteins resulted in potent apoptotic activity[23]. These disparities may be due to the inherent biological differences between the investigated cell types. Current insight also suggested that the IAP family, including cIAP-1, cIAP-2, and XIAP, inhibit apoptosis by directly inhibiting activation of effector caspases[24,25]. A shift toward death signals is the activation of effector caspases such as caspases-3 and -9, and caspase signaling is initiated and propagated by substrates such as PARP[26]. Nevertheless, it is still not known whether ZD1839-induced apoptosis is related to the downregulation of IAP family proteins. Our results suggest that ZD1839-induced apoptosis is associated with decreased expression levels of cIAP-1 and XIAP, but not cIAP-2. These results indicate that the downregulation of Bcl-2 and the IAP family might also activate caspase-3 and induce apoptosis in U937 cells in response to ZD1839.

The MAPK family proteins and PI3K/Akt pathways play critical roles in cell survival and death in many physiological and pathological settings. It is well known that the activation of the p38 MAPK and JNK pathways leads to the phosphorylation of a variety of pro-apoptotic downstream effectors, whereas the ERK and PI3K/Akt pathways are more often associated with cell survival[27,28]. In the present study we found that ZD1839 modulated the downregulation of ERK and Akt for apoptotic death. In contrast to our data, another recent study reported that combined treatment with ZD1839 and SP600125 had no effect on colony formation in bile duct carcinoma[29]. More experiments are required in order to find the relationship between ZD1839 and the JNK pathway in leukemic cells. Sumitomo *et al* also suggested that ERK, but not Akt, is involved in the mechanism by which ZD1839 exerts its chemosensitizing effect[30]. On the other hand, the inhibition of ERK or PI3K has been shown in preclinical and clinical studies to provide a potential antitumor effect in some cancer types[11]. These disparate observations regarding the nature of the signaling pathways represent the different apoptotic mechanisms associated with ZD1839 in different cellular systems. Although exceptions occur, the bulk of the evidence indicates that constitutive activation of PI3K/Akt signaling increases the survival threshold of cancer cells.

In conclusion, our findings may provide insight into one of the mechanisms to improve the efficacy of anticancer drugs and to support the idea that ZD1839 could induce apoptosis in human leukemic U937 cells through the EGFR-independent pathway. However, further studies are necessary in order to investigate what kinds of molecular pathways are related to EGFR-independent apoptosis.

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