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Molecular mechanisms of ZD1839-induced G1-cell cycle arrest and apoptosis in human lung adenocarcinoma A549 cells

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Abstract

Epithelial growth factor receptor (EGFR) has been proposed as a target for anticancer therapy. ZD1839 (Iressa) is a quinazoline derivative that selectively inhibits the EGFR tyrosine kinase activity and is under clinical use in cancer patients. However, the molecular mechanisms involved in ZD1839-mediated anticancer effects remain largely uncharacterized. In this study, exposure of human lung adenocarcinoma A549 cells to ZD1839 caused G1 arrest, and subsequently induced apoptosis. Moreover, ZD1839 increased the protein levels of $p27^{KIP1}$ and retinoblastoma-related Rb2/p130 while decreased the expression of cyclin-dependent kinase-2 (CDK2), CDK4, CDK6 and cyclin-D1, cyclin-D3. In vitro kinase assay showed that ZD1839 decreased these CDKs expression in A549 cells, leading to significantly reduce their kinase activities. In addition, ZD1839-induced death of A549 cells with characteristics of apoptosis including apoptotic morphological changes, DNA fragmentation and enhancement of TUNEL-positive cell. These events were accompanied by a marked increase of Fas protein expression, and activation of caspase-2, -3, -8. Co-treatment of cells with Fas antagonist antibody significantly blocked ZD1839-induced apoptosis. Caspase-8 and caspase-3 inhibitors, but not a caspase-9 inhibitor, were also capable of restoring cell viability. Our results indicate that downregulation of the expression and function of CDK2, CDK4, CDK6, cyclin-D1 and cyclin-D3, as well as upregulation of $p27^{KIP1}$ and pRb2/p130, are strong candidates for the cell cycle regulator that arrests ZD1839-treated A549 cells at G1 phase. Furthermore, upregulation of Fas appears to play a major role in the initiation of ZD1839-induced apoptosis, activation of caspase-8/caspase-3 cascade is involved in the execution phase of this death program.

Keywords: Apoptosis; Caspase; Cyclin; Cyclin-dependent kinase; Fas; $p27^{KIP1}$

1. Introduction

Lung cancer is the leading cause of cancer deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer patients and they are usually associated with poor prognosis [1]. In spite of new treatments, the overall five-year survival rate remains about 14% and most patients present with advanced disease [1]. Obviously, novel therapeutic strategies to improve efficacy in accord with safety are urgently needed.

Epidermal growth factor receptor (EGFR) plays an important role in the regulation of cell proliferation, differentiation, development and oncogenesis [2–4] through signal transduction. This signal transduction pathway can also lead to cell proliferation in tumor growth as well as progression of invasion and metastasis [5–7]. Many epithelial cancers, particularly carcinomas of the upper aerodigestive tract including NSCLC, especially the squamous

Abbreviations: CDK, cyclin-dependent kinase; CKI, cyclin-dependent protein kinase inhibitor; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide; NSCLC, non-small cell lung cancer; TNFRI, tumor necrosis factor receptor 1; TNFRII, tumor necrosis factor receptor 2; TUNEL, terminal transferase-mediated dUTP-fluorescein nick end-labeling

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cell carcinoma, display EGFR overexpression with or without EGFR gene amplification often associated with increased production of EGFR ligands [8,9]. Accumulating evidence indicated that overexpression of EGFR is associated with metastasis and a poor prognosis in patients with cancer [10,11]. ZD1839, a quinazoline-derived compound recognized as EGFR tyrosine kinase inhibitor [10,11], is now approved for advanced NSCLC in patients whose disease has progressed after treatment with platinum-based and docetaxel chemotherapy. Studies suggest that squamous cell carcinoma of the lung is more likely to overexpress EGFR than adenocarcinoma of the lung [12–15]. However, the level of EGFR expression in cells from which several xenografts (human NSCLC, prostate, and vulvar cancers) were derived reveals a very wide range of expression. There was no clear correlation between the level of EGFR expression and xenograft sensitivity to ZD1839. In animal studies, the level of expression of EGFR did not predict sensitivity to ZD1839 [16,17]. Later, in phase II clinical trials, adenocarcinoma showed a better response rate than squamous cell carcinoma [18]. ZD1839 has synergistic effects with some chemotherapy agents in animal studies [16,17], but no significant differences in overall survival in chemonaive patients with advanced NSCLC in phase III clinical trials [19,20]. The effect of ZD1839 was cytostatic, but higher doses increased apoptotic cell death. In oral KB cells, which overexpress EGFR, cytotoxicity were not observed at ZD1839 concentrations up to 25 μM [21]. Clinically, most of the NSCLC patients respond to ZD1839 within 14 days, a time even shorter than chemotherapy agents, which lead its effect by apoptosis. Thus the effect induced by ZD1839 would not be just cytostatic, there must be some component of cytotoxic effect, which are found in the skin biopsies of cancer patients with increased apoptotic index after ZD1839 treatment [22]. The effector mechanisms involved in ZD-1839-mediated apoptosis and cell cycle arrest remain largely uncharacterized although with clinical usefulness. In the present study, the cellular and molecular mechanisms of ZD1839 action against human cancer cells was examined in human lung adenocarcinoma A549 cells. Our results indicate that inhibition of cell cycle progression and induction of apoptotic cell death contribute to the antiproliferative effects of ZD1839 in A549 cells. These findings may be relevant to the tumor regressive effects of this compound in humans.

2. Materials and methods

2.1. Cell cultures and growth inhibition assay

Three NSCLC cell lines, A549, H1299 and CH 27, were maintained in RPMI 1640 medium and supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin), at 37 °C in a humidified atmosphere of 5% CO_2. Culture medium was changed every two days. For growth inhibition assay, cells were seeded into 12-well plates at a density of 3×10^4 cells/well. After 24 h, cells were treated with various concentrations of ZD1839 for the indicated time points. After treatment, the number of viable cells was determined by Trypan blue dye exclusion method using a hemocytometer. 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, obtained from Sigma Company) assay was also performed to examine the antiproliferative effect of ZD1839. After treatment with ZD1839, lung cancer cells were incubated with 1 μg/ml MTT for another 1–2 h, $A_{550\ nm}$ was measured.

2.2. Cell cycle analysis

Cells were treated with 0.1% dimethyl sulfoxide (DMSO as vehicle control) or with ZD1839 (10 and 25 μM) for 24 and 48 h. After incubation, cell cycle distribution was evaluated by flow cytometry analysis. Briefly, 2×10^6 cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed in 80% ethanol. These fixed cells were washed with PBS, incubated with 100 μg/ml RNase at 37 °C for 30 min, then stained with propidium iodide (50 μg/ml), and analyzed using a FACScan flow cytometer (Becton Dickinson Instruments). The percentage of cells in different phases of the cell cycle was analyzed using Cell-FIT software.

2.3. Protein preparation, immunoblotting and immunoprecipitation

Cells were cultured without or with 10 and 25 μM ZD1839 for the indicated time periods. After treatment, cell extracts were prepared as described previously; protein concentration was determined using the Bradford method. Equal amounts of sample lysate were separated by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with specific primary antibodies, including anti-human CDKs, cyclins, $p16^{INK}$, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, p53, pRb130, Fas, FasL, TRAIL receptors (DR3 and DR4), and TNF receptors (TNFRI and TNFRII) antibodies. Determinations were made using enhanced chemiluminescence kits (Amersham, ECL kits) [23]. To prepare proteins for immunoprecipitation, cells were lysed in hypotonic buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.1% NP-40, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin). Protein concentration was determined and equal amounts of precleared cell lysates were mixed with protein A-Sepharose beads (Pharmacia) and mouse anti-human CDK-specific antibodies. After 4 h incubation, samples were centrifuged and washed extensive

with hypotonic buffer. Immunoprecipitated complexes were used for kinase assay.

2.4. Kinase assay

Immunocomplexes were assayed for pRb or histone H1 kinase activity by washing twice with kinase buffer, followed by incubation in 30 μl of kinase buffer containing 20 mM Tris–HCl, pH 7.5, 5 mM EGTA, 20 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 μg histone H1 (or 1 μg pRb substrate peptide), 5 μCi [γ-^{32}P] ATP (6000 Ci/mmol; DuPont NEN), and 1 μM ATP, at 37 °C for 15 min. The reaction was terminated by the addition of 10 μl of 4× Laemmli sample buffer, followed by boiling for 10 min. After reaction, samples were subjected to SDS–PAGE, then transferred to nitrocellulose paper. The incorporation of ^{32}P was visualized by autoradiography and quantitated with a PhosphoImage (Molecular Dynamics).

2.5. Determination of ERK-MAPK status

The determination of ERK-MAPK pathway status was based on the measurement of phosphorylated p42-p44 (activated form of ERK-MAPK) by immunoblot analysis [24] performed in the presence or absence of ZD1839.

2.6. Measurement of apoptotic cell

Cells were untreated or treated with ZD1839 for indicated time point. After treatment apoptotic cells were determined by TUNEL assay according to the manufacturer's protocol, then investigated using an Olympus IX70 fluorescence microscope [25]. TUNEL positive cell was counted as apoptotic cell.

2.7. DNA fragmentation assay

After treatment, DNA was isolated as previous described [25]. Briefly, cells were lysed in buffer containing 10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.3% Triton X-100, at 4 °C for 20 min. Cell lysates were incubated with 100 μg/ml RNase and 200 μg/ml proteinase K, at 55 °C for 4 h, then extracted with phenol/chloroform, and precipitated using sodium acetate/ethanol, overnight at −20 °C. Equal amount of DNA (5 μg/sample) were electrophoresed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide. DNA was examined using a UV Transilluminator Image System (Evergreen, UK).

2.8. Caspase activity assay

A549 cells were treated with 0.1% DMSO (control vehicle) or with ZD1839 for indicated times. Cell lysate were isolated and caspase activity was measured according to the manufacturer's protocol. Briefly, 100 μg total protein was added to reaction mixtures containing fluorogenic substrate peptides specific for caspase-2 (VDVAD-AFC), caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC), and caspase-9 (LEHD-AFC), at 37 °C for 2 h. Fluorescence was determined using a fluorescence microplate reader (Thermo Labsystem, Finland) (excitation wavelength 400 nm, emission wavelength 505 nm).

2.9. Reagents

ZD1839 [4-(3-chloro-4-fluorophenylamino)-7-methoxy-6-(3-(4-morpholinyl) propoxy)-quinazoline] was provided by AstraZeneca (Cheshire, UK). ZD1839 was dissolved in DMSO for the in vitro study. Anti-cyclin-D1, anti-cyclin-D3, anti-CDK2, anti-CDK4, anti-pRb, and anti-Fas antibodies were obtained from Transduction Laboratory. Anti-p27^{KIP1}, anti-cyclin-D2 and anti-CDK6 antibodies were purchased from Santa Cruz. Anti-ERK and anti-phospho-ERK antibodies were obtained from Calbiochem. Anti-p53, anti-p21$^{CIP1/WAF1}$, antagonistic anti-Fas (clone ZB4), and agonistic anti-Fas (clone CH11) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-FasL, anti-DR3, anti-DR4, anti-TNFRI, and anti-TNFRII were obtained from BD Pharmigen. Inhibitors of caspase-2 (Z-VADVD-FMK), caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) were purchased from Kamiya (Thousand Oaks, CA).

2.10. Analysis of data

In this study, all data are presented as mean ± S.D. of 12 replicates from four separate experiments. Statistical differences were calculated using the Student's *t*-test and considered significant at the $^{**}P < 0.01$ or $^{*}P < 0.05$ level. All the figures shown in this article were obtained from at least four independent experiments with a similar pattern.

3. Results

3.1. Antiproliferative effect of ZD1839

A549 cells were treated with various concentrations of ZD1839 (0, 1, 10, 25 μM), viable cells were measured daily by the Trypan blue dye exclusion method and MTT assay. As shown in Fig. 1A, ZD1839 inhibited the growth of A549 cells in a dose-dependent manner. After 4 days incubation, the cell number of 1 and 10 μM ZD1839-treated cultures reduced approximately 30% and 50%, respectively. The growth of A549 cells was almost completely suppressed in the continuous presence of 25 μM ZD1839 for 2 days, the cell number was maintained approximately similar to that of initial seeding. The antiproliferative effect of ZD1839 was observed not only in A549 cells but also in other lung cancer cell lines. As

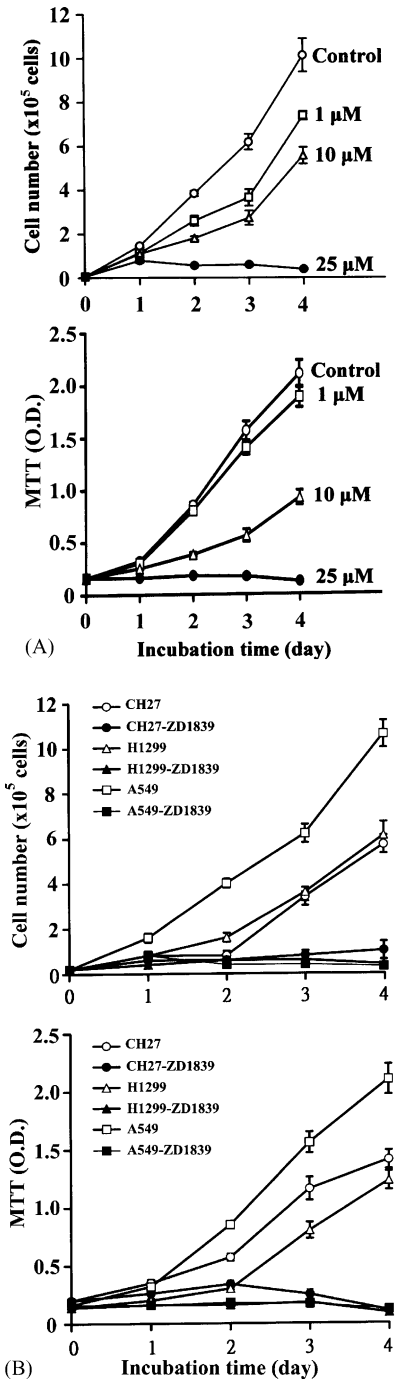

Fig. 1. Antiproliferative effect of ZD1839. (A) Dose- and time-dependent response. Human lung adenocarcinoma A549 cells were treated with various concentrations of ZD1839 (0, 1, 10, and 25 μM) for indicated time points (1, 2, 3, and 4 days). After treatment, cell number was measured by Trypan blue dye exclusion method and MTT assay. (B) ZD1839-mediated growth inhibition. H1299, CH27 and A549 cells were treatment with 25 μM ZD1839 for indicated time points (1, 2, 3, and 4 days), direct cell number was estimated and MTT assay was performed daily.

indicated in Fig. 1B, treatment with 25 μM ZD1839 caused a dramatic growth inhibition in human lung squamous carcinoma CH27 cells and large cell lung cancer H1299 cells. Similarly, the inhibition of tumor cell growth by ZD1839 was also observed using the MTT assay

Table 1
Effect of ZD1839 on the cell cycle distribution of A549 cells

Exposure time	Concentration (μM)		
	0	10	25
1 day			
SubG1	4.0	5.7	8.1
G1	49.6	63.0	70.1
S	38.9	26.5	22.0
G2/M	11.5	10.5	7.9
2 days			
SubG1	3.2	7.3	32.1
G1	58.7	64.1	83.3
S	32.1	27.1	12.9
G2/M	9.2	8.8	3.8

Note. A549 cells were treated with ZD1839 and analyzed for DNA content by propidium iodide staining. Flow cytometry data were processed using the Cell-Fit software (Becton-Dickinson). Data presented are from three independent experiments.

(Fig. 1A and B). Morphological examination of A549 cells using a phase-contrast microscope showed that the antiproliferative effect induced by low dose of ZD1839 (<10 μM) was mainly cytostatic. However, long-time persistent exposure of A549 cells to high dose of ZD1839 (25 μM) caused a cytotoxic effect, because floating dead cells were observed after 3–4 days incubation (data not shown).

3.2. Induction G1 arrest by ZD1839

To determine the effect of ZD1839 on the cell cycle progression of A549 cells, flow cytometry analysis was performed on cells treated with 0, 10, and 25 μM ZD1839 for 1 and 2 days. As shown in Table 1, administration of ZD1839 caused a time- and concentration-dependent increase in the proportion of cells in the G1 phase, with a corresponding decrease in the proportion of cells in both S and M phases in comparison with control cultures. These results indicated that ZD1839 mediated a prolongation of cell cycle progression in G1 phase in A549 cells.

3.2.1. Regulation of cell cycle G1-related proteins by ZD1839

Cell cycle progression is regulated through the positive and negative cell cycle regulatory molecules, such as CDKs, cyclins, CDK inhibitors (CDKI), p53 and pRb [26]. To elucidate the specific cell cycle regulatory proteins responsible for the G1 block mediated by ZD1839 in A549 cells, we focused upon the proteins involved in the G1 phase of the cell cycle. Protein extracts were prepared from cells treated with 25 μM ZD1839 for 1, 2 and 3 days. Western blot analysis was performed using antibodies specific for CDKs (CDK 2, 4, and 6), cyclins (cyclin D1, D2, D3, and E), CDKIs ($p21^{CIP1/WAF1}$, $p27^{KIP1}$), p53, pRb and pRb2 proteins. We found that treatment with ZD1839 resulted in a significant reduction in CDK2,

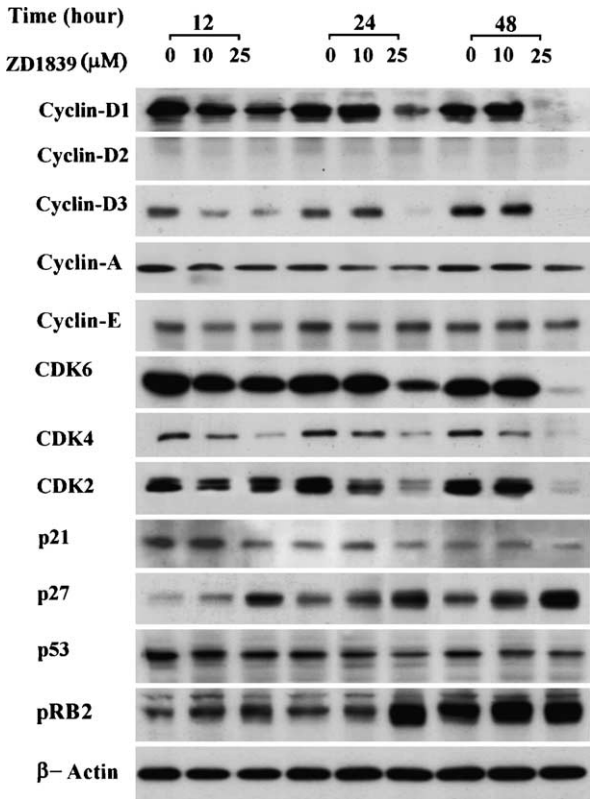

Fig. 2. Regulation of G1-related proteins by ZD1839. A549 cells were treated with various concentrations of ZD1839 (0, 10, and 25 μM) for indicated time periods (12, 24 and 48 h). After treatment, G1-related proteins were analyzed by Western blot using specific antibodies.

CDK4, CDK6, cyclin-D1 and cyclin-D3 protein expression (Fig. 2). In contrast, the levels of $p27^{KIP1}$ and pRb2/p130 proteins drastically increased upon ZD1839 treatment. However, the cyclin-E, $p21^{CIP1/WAF1}$, pRb1, and p53 proteins were constitutively expressed, and no changes in their levels were observed in cell growth arrested by ZD1839 treatment (Fig. 2). In A549 cells, no cyclin-D2 protein could be detected. These results imply that the down-regulation of CDK2, CDK4, CDK6, cyclin-D1, and cyclin-D3 protein expressions may be responsible for the G1 growth arrest induced by ZD1839. In addition, upregulation of $p27^{KIP1}$ and pRb2 proteins may also be involved in ZD1839-triggered G1 cell cycle block.

3.3. Modulation of CDK2-, CDK4-, and CDK6-associated kinase activities by ZD1839

To verify whether the ZD1839-induced downregulation of CDK2, CDK4, and CDK6 were associated with changes in the kinase activities of various cyclin-CDK complexes, in vitro kinase assay were performed. When histone H1 or pRb was used as substrate in immunoprecipitation experiments performed with antibodies against CDK2, CdDK4, and CDK6, lysates from cells treated with ZD1839 at 0, 10 and 25 μM for 48-h showed a marked decrease in kinase activities (Fig. 3). At 25 μM ZD1839 treatment, the

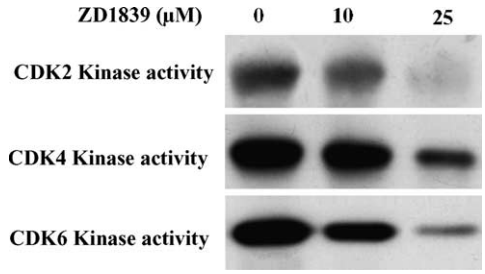

Fig. 3. Modulation of G1-related CDK activities by ZD1839. A549 cells were treated with 0, 10, and 25 μM of ZD1839 for 48 h. After incubation, cell extracts were immunoprecipitated with anti-CDK2, anti-CDK4 and anti-CDK6 antibodies. The immunocomplexes were analyzed with kinase assay for activity toward histone H1 (for CDK2), or toward pRb fragment (for CDK4 and CDK6). Quantitation was by Phospho-Imager and fold activity was calculated relative to that found in vehicle-treated control cultures.

CDK2-, CDK4-, and CDK6-associated kinase activities were significantly decreased after 48-h treatment (Fig. 3). These results demonstrated that ZD1839-mediated decrease of CDK2, CDK4 and CDK6 protein levels was consistent with the reduction in their kinase activities.

3.4. Effect of ZD1839 on ERK-MAPK

Inhibition of EGFR tyrosine activity should prevent activation of EGF-mediated downstream signals. To correlate the effect of ZD1839 on growth inhibition with EGF-dependent ERK-MAPK signaling event, we examined the effect of ZD1839 on the expression and function of ERK-MAPK (p42-p44). As shown in Fig. 4, ERK1 and ERK2 were constitutively activated in A549 cells as measured by immunoblotting of cell lysates with phospho-ERK antibody. However, treatment of A549 cells with ZD1839 strongly reduced the levels of phosphorylated ERK-MAPK (p42-p44), but did not alter the total ERK-MAPK protein levels (Fig. 4). Complete inhibition of ERK-MAPK activation was observed after 25 μM ZD1839 treatment. This result indicated that ZD1839 could inhibit the activation of ERK-MAPK but did not affect their protein expression in A549 human lung adenocarcinoma cells.

3.5. ZD1839 induced apoptotic cell death in A549 cells

After continuous incubation of A549 cells with 25 μM ZD1839 for 48–72 h, significant cytotoxic effect was observed. To investigate whether cytotoxic effect of ZD1839 was due to induction of apoptosis. DAPI staining and TUNEL assay revealed induction of apoptosis in ZD1839-treated A549 cells (data not shown). Further demonstration of apoptosis was shown by the typical ‘‘DNA laddering’’ pattern of DNA fragmentation in ZD1839 treatment (data not shown).

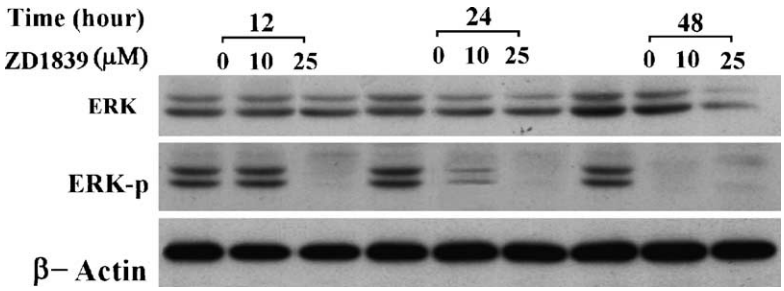

Fig. 4. Inactivation of ERK-MAPK by ZD1839. A549 cells were treated with 0, 10, and 25 μM of ZD1839 for indicated time periods (12, 24 and 48 h). The cellular levels of total ERK-MAPK and phosphorylated ERK-MAPK were evaluated by Western blot analysis using anti-ERK antibody or anti-phospho-ERK specific antibody.

3.6. Effects of ZD1839 on Bcl-family proteins

It is well documented that Bcl-2 family members can inhibit or promote apoptosis [27]. Western blot analysis showed that ZD1839 had no effect on the level of Bcl-2, Bcl-X_L, Bax, Bad, Bak, or Bid protein (Fig. 5A). Overexpression of Bcl-2 protein (by adeno-Bcl-2 viral vector infection) increased the expression of Bcl-2 protein in A549 cells, but did not protect of A549 cells from apoptosis induced by ZD1839 (Fig. 5B). These results indicated that Bcl-2 family proteins might be not involved in ZD1839-induced apoptosis.

3.7. Involvement of Fas signaling in ZD1839-induced apoptosis

To study the involvement of death-receptor proteins in ZD1839-induced apoptotic process, Western blot analysis was performed. ZD1839 treatment resulted in increase of the expression level of Fas protein (Fig. 6A). However, the protein levels of FasL, TRAIL receptors (DR3 and DR4), and TNF receptors (TNFRI and TNFRII) did not alter in ZD1839-treated cells (data not shown). To determine whether ZD1839 induces apoptosis through the activation of Fas receptor signaling, both agonistic (clone CH11) and antagonistic antibody (clone ZB4) were used. Co-exposure of A549 cells to ZD1839 and agonistic anti-Fas antibody showed a synergistic effect in the induction of apoptosis (Fig. 6B). In addition, the apoptosis-inducing effect of ZD1839 was drastically abolished by antagonistic anti-Fas antibody. These finding indicated that ZD1839-induced apoptosis in A549 cells might be mediated, at least in part, by the Fas/FasL signaling pathway.

3.8. Requirement of caspase-3 and caspase-8 activation during ZD1839-induced apoptosis

The activity of caspases in A549 cells after ZD1839 treatment was measured by using their specific fluorogenic substrates. Four synthetic oligopeptides were used: Ac-VDVAD-AFC for caspase-2; Ac-DEVD-AFC for caspase-3, -7, and -10; Ac-LETD-AFC for caspase-8; Ac-LEHD-

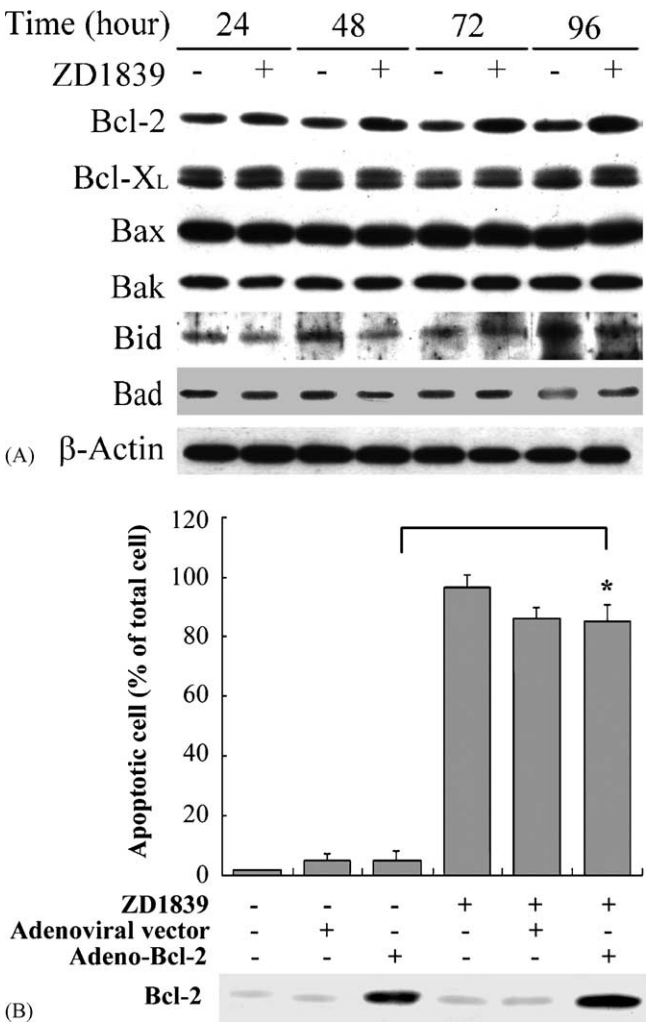

Fig. 5. Bcl-2 family proteins were not involved in ZD1839-induced apoptosis. (A) Expression of Bcl-2 family proteins. A549 cells were treated with 25 μM ZD1839 for indicated time periods. Total protein was extracted; the levels of Bcl-2, Bcl-X_L, Bax, Bad, Bak, and Bid were analyzed by Western blotting. (B) Overexpression of Bcl-2 did not block ZD1839-induced apoptosis. Cells were infected with Adv-Bcl-2 and Adv-vector for 16 h, and then treated with 25 μM ZD1839 for another 72 h, apoptotic cells were determined by TUNEL assay. The expression level of Bcl-2 protein was detected by Western blot analysis. $^{*}P < 0.05$, compared with the 25 μM ZD1839-treated group.

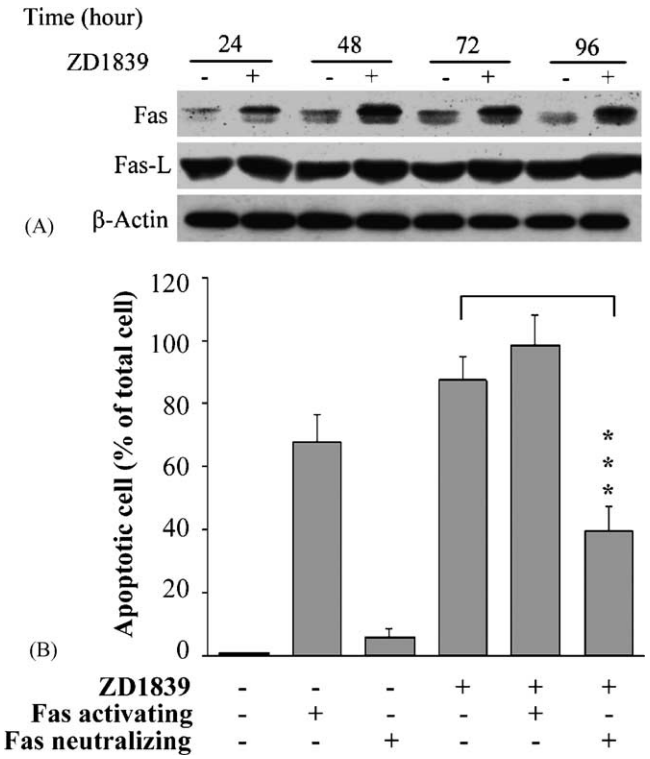

Fig. 6. Involvement of Fas signaling in ZD1839-triggered apoptotic death. (A) Protein expression. Cells were treated with 25 μM ZD1839 for indicated time points. After treatment, total protein was isolated. The levels of Fas, FasL, TRAIL receptor (DR3 and DR4), and TNF receptor (TNFRI and TNFRII) were detected by Western blot using specific antibodies. β-Actin was used as an internal loading control. (B) The effect of agonistic or antagonistic anti-Fas antibody on ZD1839-induced apoptosis. Combined treatment of A549 cells with agonistic or antagonistic anti-Fas antibody and ZD1839 for 72 h, and then apoptotic cell was determined by TUNEL assay. $^{***}P < 0.001$, compared with the 25 μM ZD1839-treated group.

AFC for caspase-9. Cell lysates were prepared after ZD1839 treatment at various time intervals. As shown in Fig. 7A, caspase-8 and -3 was clearly activated at 36 h, early than other caspases and before the onset of apoptosis, and maximal activity was seen at 48 h, before the maximal levels of apoptosis achieved. Caspase-2 was slightly activated after 48 h ZD1839 treatment. However, caspase-9 was not activated in ZD1839-treated A549 cells. The activation of caspase-8 and -3 were also confirmed by detection of cleaved active fragments by Western blot (Fig. 7B). To define whether a particular caspase plays the crucial role in ZD1839-induced apoptosis, specific caspase inhibitors were used to address this question. All cell-permeable caspase inhibitors used below were nontoxic to A549 cells under the concentrations applied. As depicted in Fig. 7C, the caspase-8 or caspase-3 inhibitor protected the cells from death due to ZD1839 treatment. By contrast, the inhibitor of caspase-2 or -9 failed to inhibit ZD1839-induced apoptosis. These observations indicated that caspase-8 and caspase-3 activation plays a crucial role in ZD1839-triggered apoptotic process in A549 cells.

Our results imply that ZD1839-induced apoptosis can be executed in a Fas-dependent caspase-8 activation pathway.

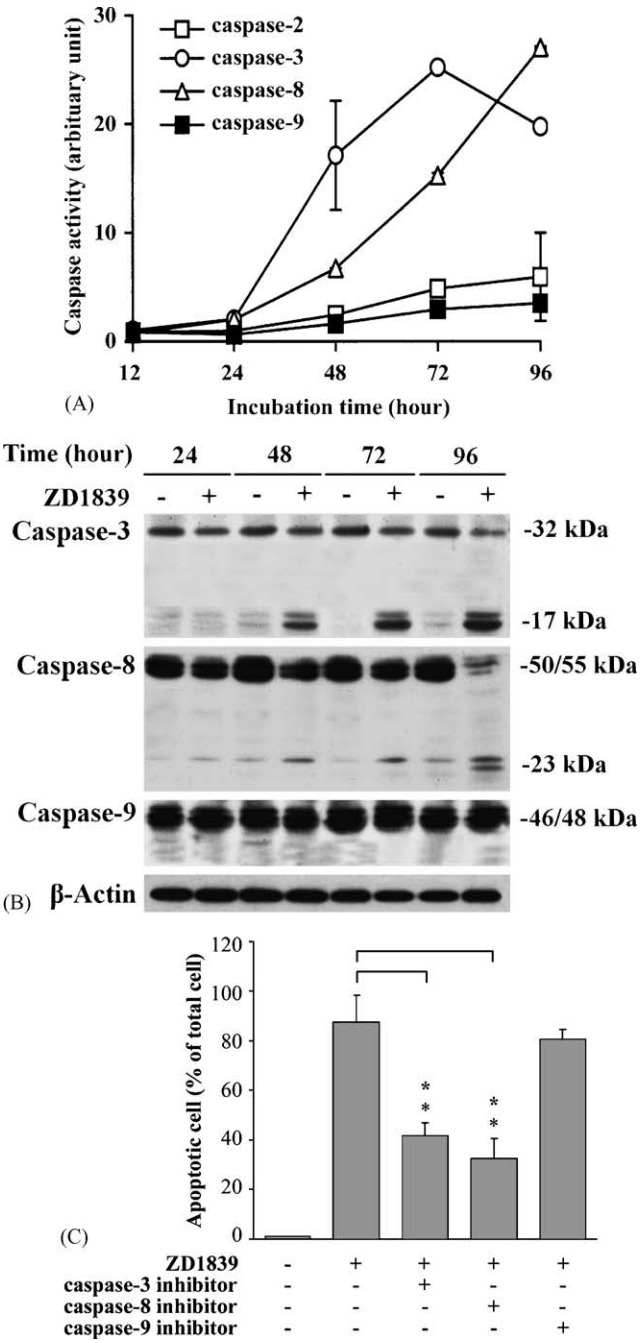

Fig. 7. Requirement of caspase-3 and -8 activation during ZD1839-induced apoptosis. A549 cells were treated with 25 μM ZD1839 for indicated time periods. After treatment, cell lysate was isolated, (A) the activities of caspases were measured using specific fluorogenic peptide substrates, or (B) analyzed for proteolytic active fragment by Western blot. (C) Inhibition of ZD1839-induced apoptosis by caspase inhibitor. Combined treatment of A549 cells with specific caspase inhibitor and ZD1839 for 72 h, and then apoptotic cell number was determined by TUNEL assay. $^{**}P < 0.01$, compared with the 25 μM ZD1839-treated group.

We expanded our study to other two human lung cancer cell lines, H1299 and CH27. In both cell lines, upregulation of Fas protein (Fig. 8A) and activation of caspase-8 cascade (Fig. 8B and C) were required for ZD1839-induced apoptosis.

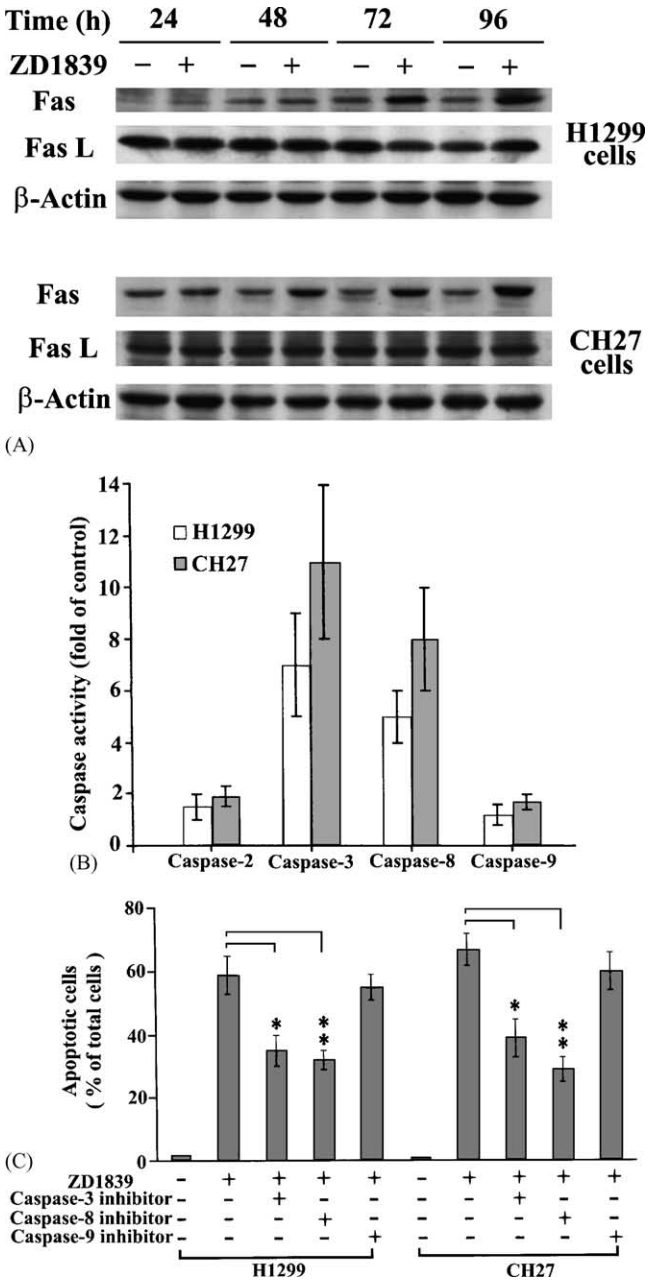

Fig. 8. Upregulation of Fas and activation of caspase-8 cascade was involved in ZD1839-induced apoptosis in both H1299 and CH27 lung cancer cell lines. (A) Upregulation of Fas in ZD1839-treated cells. H1299 and CH27 cells were treated with 25 μM ZD1839 for indicated time points. The protein levels of Fas and FasL were measured by Western blot analysis. β-Actin was used as an internal loading control. (B) Activation of caspase-8 and -3 by ZD1839. H1299 and CH27 cells were treated with 25 μM ZD1839 for 72 h, after treatment, caspase activities were measured. (C) Caspase inhibitor blocked ZD1839-induced apoptosis. Combined treatment of both cell lines with specific caspase inhibitor and ZD1839 for 72 h, and then apoptotic cell number was determined by TUNEL assay. $^{*}P < 0.05$; $^{**}P < 0.01$.

4. Discussion

As the median time to symptom improvement to ZD1839 treatment was 8 days, most patients who experienced a partial response or stable disease also had an improvement in their disease-related symptoms [28]. Clinically, big lung tumors can shrink to small ones in responsive patients, within such a time even shorter than chemotherapy agents. This effect would not be just cytostatic, there must be some component of cytotoxic effect by ZD1839. We observed evident apoptosis in high level (25 μM) ZD1839 in A549 cell line and other NSCLC cell lines. This level seems much higher than the plasma concentrations of the patients of phase I studies [29] and those published by Ciardiello et al. [16]. Conversely, other investigators using similar experimental conditions as ours have reported on ZD1839 cytotoxic values very close to those used in this study [21,24,30,31]. Magne et al. suggests that the used ZD1839 concentrations were dictated by the experimental conditions and, as compared to in vivo conditions, to a relatively short exposure of the tumor cells to the drug (given once) [24]. It is normal that, in vivo, when tumor cells are continuously exposed to the parent drug, the observed concentration may be lower; in vivo there is also the possible presence of active metabolites carrying a part of the activity, such metabolites are obviously not present in the in vitro condition. On the other hand, for cell survival in vitro, the presence of serum in the culture medium is needed and this situation brings growth factors which stimulate the drug pathway which is targeted by the drug. Although ZD1839 consistently inhibited EGFR phosphorylation and modulated the activity of proteins downstream in the EGFR pathway in skin samples taken from patients [22], there are few data about the degree of inhibition of the EGFR pathway in tumor tissue. Pharmacodynamic effects measured in skin may not predict for similar effects in tumors. In this study, we try to evaluate the apoptosis induced by ZD1839, which may explain the rapid response of the NSCLC. Previous studies have shown that ZD1839 has numerous effects on tumor cells including cell cycle arrest, increase in apoptosis and reduction in cell proliferation [16,17]. Interestingly, here we found that following treatment with ZD1839 (at concentrations >10 μM) for more than 48 h resulted in an apoptotic cell death in A549 cells. Induction of apoptosis by ZD1839 has been reported in several cell lines derived from carcinomas of different histotypes [16,32–34]. Ciardiello et al. had reported that ZD1839-mediated antiproliferative effect was mainly cytostatic in several human cancer cell lines, but also caused an apoptotic cell death at high dose treatments [16]. Similarly, our results also demonstrated that low doses (1 μM) of ZD1839 induced cytostatic effect; however, long-time high dose (25 μM for 72 h) of ZD1839 treatment resulted in apoptosis in human adenocarcinoma A549 cells. These results contrast with previous study [35], showing ZD1839 failed to promote apoptosis, either in vitro or in vivo. The possible reasons for this discrepancy may be due to the differences of dose, incubation time, and cell type.

Our cell cycle analyses revealed prominent G1 arrest of A549 cells after exposure to 25 μM of ZD1839, and this

was accompanied by significantly decrease in S phase. This result is consistent with that of other investigators, who showed that ZD1839 induced G1 arrest in several types of human cancer cell lines [36]. Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle regulatory proteins (including cyclins, CDKs, CKIs, p53, and pRb) provides an important mechanism for inhibition of growth [25,37]. In the present study, we showed that ZD1839 strongly downregulated the expression of CDK2, CDK4, CDK6, cyclin-D1 and cyclin-D3 proteins, reduced levels of these G1-related CDKs and cyclins in ZD1839-treated cells, may facilitate cell cycle blocking in mid-G1 and G1/S border. Interestingly, to our knowledge, this is the first demonstrating that downregulation of CDK4, CDK6, and cyclin-D3 are the mediators in the antiproliferative effect of ZD1839 on human cancer cells.

The activity of cyclin/CDK complexes is also negative regulated by binding to cyclin-dependent kinase inhibitors (CKIs) [38]. These CKIs are required for proper cell cycle arrest in response to mitogen deprivation and genotoxic stress [38]. The physiological role of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ have been linked to inhibition of G1-related CDKs kinase activities [38]. Previous studies demonstrated that the ZD1839-induced G1 arrest was associated with an elevation of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ and a reduction of CDK2 activity in human head and neck cancer cell lines [39,40]. Similar findings are also noted by Budillon [41] and they suggested that $p27^{KIP1}$ plays a key role in ZD1839-induced cell cycle perturbation by decreasing CDK2 activity and leading to G1 growth arrest. However, in our study, a marked increase in the protein level of $p27^{KIP1}$ was detected in ZD1839-treated A549 cells, while the levels of $p21^{CIP1/WAF1}$ and p53 proteins were not changed. This result suggests that $p27^{KIP1}$ may play an important role in the control of the G1/S transition in ZD1839 treated A549 cells. The retinoblastoma (Rb) gene products, pRb, p107 and pRb2/p130, are negative regulators of the transition between the phases G1 and S of the cell cycle [42,43]. Here, we showed that ZD1839-induced G1 growth arrest was accompanied by upregulation of pRb2/p130, suggesting pRb2/p130 may also involve in ZD1839-mediated G1 blockade in A549 cells. Similar results were obtained on treatment of human cancer cells with another EGFR tyrosine kinase inhibitors. CP358,774 induced G1 arrest in human colorectal carcinoma DiFi cells. This was accompanied by a marked accumulation of hypophosphorylated pRb and $p27^{KIP1}$ proteins, which may contribute to the G1 cell cycle block [44]. AG1478 inhibited A431 cell proliferation by upregulation of $p27^{KIP1}$, downregulation of cyclin-D1, and hypophosphorylation of the pRb [45]. These observations imply that upregulation of $p27^{KIP1}$ and accumulation of hypophosphorylated pRb-related proteins are required for the antitumor action of EGFR kinase inhibitors. Furthermore, our findings indicate that ZD1839-mediated antiproliferative action does not require functional p53 because p53 and its downstream molecule $p21^{CIP1/WAF1}$ were not regulated by ZD1839, and cells lacking functional p53 (H1299) exhibit a response to ZD1839 treatment similar to cells with functional p53 (CH27 and A549), thus expanding the spectrum of malignancies that might potentially respond to ZD1839.

In addition, ZD1839 treatment reduced the activation status of ERK-MAPK. ERK lies on a signaling pathway that stimulates cell cycle progression via induction of immediate early genes such as c-*fos* and through effects on cell cycle regulators such as cyclin-D and $p27^{KIP1}$ [46–48]. The data present here showed that A549 cells exhibited an intrinsically active ERK-MAPK; however, treatment with ZD1839 resulted in a strong inhibition of this intrinsic ERK-MAPK activation. Data shown in Figs. 1 and 4 indicate that the reduction in cell growth coincides with the inhibition of ERK-MAPK activation in ZD1839-treated A549 cells. Therefore, the inhibition of ERK activity that we detected in A549 cells as a result of EGFR inhibition by ZD1839 and may be instrumental in the reduction in growth rate seen in A549 cells.

Apoptosis has been described as multiple pathways converging from numerous different initiating events and insults [49]. Numerous studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, gemcitabine, etoposide, taxol, etc. [49]. Accumulating evidence showed that efficacy of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [49]. Morphological changes of apoptosis are considered the results of complex cellular biochemical pathways [50]. In mammals, apoptosis is a result of the proteolysis of various cellular components initiated by activated caspases (a family of cysteine proteases) [51]. The current study showed that ZD1839-induced apoptotic cell death was accompanied by marked activation of caspase-3, and -8. Inhibitors of caspase-8 and -3 significantly abolished ZD1839-triggered apoptotic death; suggest that activation of caspase-8/caspase-3 cascade is required for ZD1839-induced apoptosis in A549 cells. Since caspase-8 or caspase-3 inhibitor has partial effect to protect cells, indicating other pathway might be involved in ZD1839-induced cell death.

Many lines of evidence indicated that the Bcl-2 family proteins play important roles in the control of mitochondrial integrity, since the loss of mitochondrial membrane potential leads to the release of intermembrane molecules (including cytochrome *c*, apoptotic-inducing factor, caspase-2 and caspase-9) into cytosol and induces apoptotic cell death [52,53]. Recently, ZD1839 was found to induce apoptosis by activating the pro-apoptotic protein Bad in non-transformed and cancer mammary epithelial cells [54]. This study showed that ZD1839 prevents MAPK-dependent phosphorylation of Bad at serine residues 112 and 115. Serine phosphorylation inactivates Bad by

sequestering it in the cytosol and inhibiting its interaction with Bcl-X_L [55]. In this study, we found that ZD1839 blocked the activation of MAPK-ERK in A549 cells, but did not alter the expression level and phosphorylation status of Bad protein (data not shown), suggesting that ZD1839-induced apoptosis did not involve the antagonizing of MAPK-mediated phosphorylation of Bad. In addition, we also found that there were no changes in the levels of Bcl-2, Bcl-X_L, Bax, Bak, and Bid proteins following ZD1839 treatment, the release of cytochrome *c* from mitochondria to cytosol (data not shown) and activation of caspase-9 (Fig. 7) could not be detected. In addition, overexpression of Bcl-2 protein by infection of adeno-Bcl-2 viral vector could not prevent ZD1839-triggered apoptotic cell death, suggesting Bcl-2-family-dependent mitochondria pathway was not involved in ZD1839-triggered apoptotic process in A549 cells. Conversely, Janmaat et al. reported that ZD1839-induced cell death involves a Bcl-2-dependent mitochondria pathway [56]. These differences may be due to the biological differences between the investigated cell types.

Some reports have shown that the activation of the Fas/FasL system may be one of the mechanisms responsible for drug-induced apoptosis in a variety of cancer cells of different histotype [57]. Our observations provide the first demonstration that ZD1839 induces apoptosis in all three tested human lung cancer cell lines via the activation of Fas/caspase-8 pathway. This conclusion can be drawn from the following experimental data: (1) lung cancer cells treated with ZD1839 showed an increase in the expression level of Fas protein before apoptosis occurred; (2) co-treatment with activating anti-Fas antibody and ZD1839 caused a synergistic induction of apoptosis; (3) ZD1839-triggered apoptotic effect can markedly be inhibited by antagonistic anti-Fas antibody and (4) ZD1839-treated lung cancer cells showed the activation of caspase-8 which has been found to be a prominent signaling caspase involved in initiation of apoptosis by Fas [57]. Moreover, co-exposure to a caspase-8 inhibitor (Z-IETD-FMK) and ZD1839 significantly inhibited ZD1839-triggered apoptosis in all three tested lung cancer cell lines. Previous study demonstrated that inhibition of EGFR pathway induces a death receptor-mediated apoptosis in human breast cancer cells [58]. Kalas et al. indicated that inhibition of MEK/ERK pathway induces Fas expression and apoptosis in lymphomas [59]. In agreement with these in vitro findings, the ZD1839-mediated ERK inactivation may contribute to the upregulation of Fas in our experimental conditions. The exact mechanism of ZD1839-induced Fas expression remains to be explored.

Several studies have shown that blockade of the EGFR by ZD1839 produces a significant reduction in the activation of MAPK in the cancer cells, no direct correlation has been established between the efficacy of the therapy and the reduction in the activation of either MAPK in both clinical and experimental studies [60,61]. Since the levels of expression of EGFR are not related to the response to ZD1839, it is clear that the mode of action of ZD1839 is more complex than simply blocking the tyrosine kinase activity of the EGFR [60,61]. At this stage, how does ZD1839 regulate Fas protein and activate caspase-8 cascade? The possible relationship between the inactivation of MAPK-ERK and activation of Fas system in ZD1839-triggered apoptotic process remain unclear and are the subject of ongoing research in our laboratory.

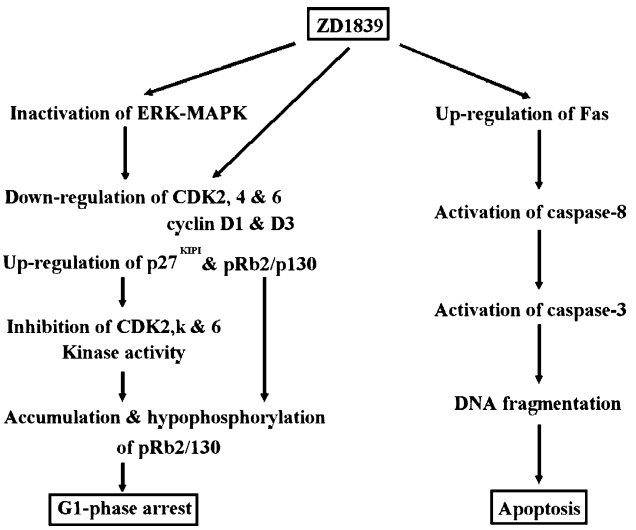

Fig. 9. Schematic diagram of the G1-phase arresting and apoptotic signaling pathway induced by ZD1839 in human lung carcinoma A549 cells.

Our observations indicate that inhibition of cell cycle progression and induction of apoptotic cell death contribute to the antiproliferative effects of ZD1839 in human lung adenocarcinoma A549 cells. The most likely mechanism underlying the ZD1839-induced growth arrest involves an initial inhibition of intrinsic EGFR-dependent ERK-MAPK activation that led to the reduction of the G1-related CDKs (CDK6, CDK4, and CDK2) and cyclins (cyclin-D1 and cyclin-D3), overexpression of $p27^{KIP1}$ protein, and consequently decreased CDK6, CDK4 and CDK2 kinase activities and accumulation of hypophosphorylated pRb2/p130, that ultimately arrested A549 cells in the G1 phase (Fig. 9). Furthermore, the increase of expressing level of Fas protein and activation of caspase-8/caspase-3 cascade may be the molecular mechanism through which ZD1839 induces apoptosis (Fig. 9) in all three tested lung cancer cell lines.

Acknowledgements

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