

Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small Cell Lung Cancer Cells: Limited Antiproliferative Effects and Absence of Apoptosis Associated with Persistent Activity of Extracellular Signal-regulated Kinase or Akt Kinase Pathways

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ABSTRACT

The epidermal growth factor receptor (EGFR) is an important novel target for anticancer therapy. In this study, we examined the molecular mechanisms that underlie the antitumor effects of the anti-EGFR monoclonal antibody C225 (Cetuximab) and the selective EGFR tyrosine kinase inhibitor ZD1839 (Iressa; AstraZeneca) in non-small cell lung cancer (NSCLC) cell lines. Cell growth, assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, was inhibited at low concentrations of ZD1839 and C225 in control A431 cells, whereas the NSCLC cell lines were comparatively more resistant. In A431 cells, but not in the NSCLC cells, ZD1839 treatment resulted in a modest increase in DNA fragmentation, the externalization of phosphatidyl serine, and the activation of caspase-3, known markers of apoptotic cell death. However, poly(ADP-ribose) polymerase cleavage was not detected, and caspase inhibition by carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone partially reduced ZD1839-generated DNA fragmentation. Overexpression of the antiapoptotic protein Bcl-2 in A431 cells suppressed the cytotoxicity upon anti-EGFR treatment. These results thus demonstrate that the toxic effect of ZD1839 in A431 cells is caused by a form of cell death that involves a mitochondrial step and is, at least in part, dependent on caspase activation. EGFR expression levels showed no significant correlation with sensitivity to ZD1839 and C225. Evaluation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase and phosphatidylinositol 3'-kinase/Akt pathways showed considerable inhibition of these pathways by ZD1839 and C225 in A431 cells, whereas one or both of these pathways remained

active upon anti-EGFR treatment in NSCLC cells. In addition, treatment with specific inhibitors of mitogen-activated protein kinase kinase or phosphatidylinositol 3'-kinase resulted in a smaller effect on proliferation than simultaneous treatment with both inhibitors, whereas induction of apoptosis was observed only when both pathways were blocked. Together, these data suggest that persistent activity of either of these signaling pathways is involved in the lack of sensitivity of NSCLC cell lines to EGFR inhibitors.

INTRODUCTION

High expression of the EGFR² and/or its ligands is common in several tumor types, including head and neck cancer, breast cancer, ovarian cancer, and NSCLC, and correlates with more aggressive disease, resistance to chemotherapy, and a poor prognosis (1). Moreover, in lung tumors and other tumors, coexpression of EGFR and its ligand, transforming growth factor α , is common, suggesting an important role for the EGFR/transforming growth factor α autocrine loop in cancer (1, 2).

EGFR is a M_r 170,000 transmembrane protein consisting of an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Upon ligand binding, the receptor dimerizes, either as a homodimer or as a heterodimer with other members of the ErbB family of receptor tyrosine kinases, preferably ErbB2 (HER2), and undergoes autophosphorylation at specific tyrosine residues of the intracellular domain. The phosphorylated tyrosine residues then serve as docking sites for proteins such as Grb2, Shc, and phospholipase C, which, in turn, activate downstream signaling pathways, including the Ras/MEK/Erk and the PI3K/Akt pathway, which regulate transcription factors and other proteins involved in biological responses such as proliferation, cell motility, angiogenesis, cell survival, and differentiation (3, 4).

Two main strategies have been developed to target the EGFR and block its activation in cancer cells (5, 6). Mabs

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² The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; NSCLC, non-small cell lung cancer; MEK, mitogen-activated protein kinase kinase; Erk, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3'-kinase; Mab, monoclonal antibody; PS, phosphatidyl serine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; zVAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone; PI, propidium iodide; 7-AAD, 7-amino-actinomycin D; DEVD, benzyloxycarbonyl-Asp-Glu-Val; AFC, 7-amino-4-trifluoromethyl coumarin; GSK3 β , glycogen synthase kinase-3 β .

against the extracellular domain of EGFR compete with ligand for receptor binding, thereby preventing kinase activation. An example of this is the human-mouse chimeric Mab C225 that has a high affinity for EGFR and is currently in Phase II and III clinical trials in head and neck cancer, colorectal cancer and other tumor types (7). Other promising anti-EGFR agents are EGFR tyrosine kinase inhibitors that prevent autophosphorylation of EGFR by physical interaction with its intracellular kinase domain. ZD1839 (Iressa; AstraZeneca) is a p.o. active, selective EGFR tyrosine kinase inhibitor (8) that is currently in Phase II-III clinical trials in patients with NSCLC, among several tumor types (9–11).

Various preclinical studies have demonstrated antitumor effects of C225 and ZD1839 in a variety of cell types and mouse xenografts as single agents and in combination with other anticancer therapies, particularly chemotherapeutic agents and radiation (12–15). As single agents, the EGFR antagonists induce *in vitro* growth inhibition and, in some cell lines, apoptosis (16, 17).

To define potential markers that could predict the outcome of anti-EGFR treatment, we investigated the molecular mechanisms that underlie the antitumor effects of the EGFR antagonists C225 and ZD1839 in NSCLC cells. We used a panel of four NSCLC cell lines and the highly EGFR-expressing A431 cell line and determined *in vitro* cytotoxic and cytostatic effects after exposure to ZD1839 or C225. The EGFR inhibitors induced effective growth inhibition of A431 cells, whereas all of the NSCLC cell lines were more resistant. Cytotoxic effects were only observed in the A431 cell line, in which the role of apoptosis was further investigated. Protein expression levels of neither EGFR nor ErbB2 correlated with sensitivity to EGFR antagonists. In addition, the activity of kinase pathways downstream of the EGFR via MEK/Erk and PI3K/Akt was determined after treatment with EGFR inhibitors, showing persistent activity of at least one of these pathways in the NSCLC cell lines. Treatment with a combination of specific chemical inhibitors targeting MEK and PI3K resulted in the induction of apoptosis and effective inhibition of cell growth. Together, the results indicate that persistent activity of the MEK/Erk and PI3K/Akt kinase pathways can contribute to resistance of NSCLC cells to EGFR inhibitors.

MATERIALS AND METHODS

Chemicals. Anti-EGFR Mab C225 was kindly provided by Merck (Darmstadt, Germany) at a concentration of 2.0 mg/ml. Kinase inhibitors were provided as pure substances and diluted in DMSO. ZD1839 was a kind gift from AstraZeneca (Macclesfield, United Kingdom). U0126 and LY294002 were purchased from Cell Signaling Technology (Beverly, MA). The broad caspase inhibitor zVAD-fmk was purchased from Enzyme Systems (Livermore, CA). The stock solution of cisplatin (Bristol-Meyers Squibb, Woerden, the Netherlands) was made in PBS.

Cells and Culture Conditions. RPMI 1640 (containing 2 mM L-glutamine) and DMEM were used as culture media and were supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Breda, the Netherlands), 50 IU/ml penicillin, and 50 µg/ml streptomycin. The human NSCLC cell lines NCI-

H460 (referred to henceforth as H460), NCI-H1703 (referred to henceforth as H1703), and A549 were cultured in RPMI 1640; the human NSCLC cell line SW1573 and the vulvar squamous cell carcinoma cell line A431 were cultured in DMEM. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Cells from exponentially growing cultures were used in all experiments.

Cloning, Retroviral Transduction, and Selection of Stable Cell Lines. The DNA sequence encoding Bcl-2-FLAG was amplified with PCR using the pEFFLAGBCL2pGKpuro vector, which was a kind gift of Dr. A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia; Ref. 18), as template. The PCR fragment was inserted in the retroviral vector pLNCX2 (Clontech, Palo Alto, CA) using *Hind*III and *Bgl*II restriction sites, and the products were verified by sequencing. To make stable retrovirus-producing cells, the packaging cell line PT67 was transfected with 5–10 µg of cDNA using Superfect reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Transfected cells were grown in Geneticin-containing medium, and resistant colonies were selected and expanded. The stable transfected cells were then grown in medium without Geneticin for 72 h, and subsequently, the supernatant containing the virus was harvested and filtered through a 0.45 µm filter. After addition of hexadimethrine bromide (Polybrene; Sigma, St. Louis, MO) to a final concentration of 8 µg/ml, it was used to infect A431 cells. After 24 h, the virus-containing medium was removed, cells were selected in medium containing Geneticin, and resistant colonies were expanded. Expression of FLAG-Bcl-2 was confirmed by Western blotting using the M2 antibody that recognizes the FLAG epitope (Stratagene, La Jolla, CA).

Growth Inhibition Assay. For growth inhibition assays, 1×10^4 cells were plated into flat-bottomed 96-well plates (Costar, Corning, NY). After 24 h, various concentrations of the indicated drug were added, and the cells were incubated for an additional 72 h. Subsequently, 10% (v/v) of a solution of 5 mg/ml MTT (Sigma) was added to each well and incubated for 3 h at 37°C. Plates were centrifuged for 5 min at 1000 rpm, and the medium was carefully discarded. The formed formazan crystals were dissolved in 100 µl of DMSO, and absorbance was determined at 540 nm using a Spectra Fluorimeter (Tecan, Salzburg, Austria). Absorbance values were expressed as the percentage of the untreated controls, and the concentration of ZD1839 resulting in 50% growth inhibition (IC₅₀) was calculated. For C225 treatment, the percentage of maximal inhibition of growth was determined at a concentration of 10 µg/ml because no additional growth inhibition was observed at higher concentrations of the antibody, presumably due to saturation of EGFRs.

Clonogenic Assay. Three hundred cells/well were seeded in triplicate into 6-well plates. Twenty-four h later, the medium was replaced with medium containing ZD1839 or C225 at the indicated concentrations. After 72 h, the drugs were removed, and cells were washed twice with PBS and allowed to grow in normal medium for 7 days. Finally, cells were stained with 0.1% crystal violet in PBS for at least 30 min at room temperature, and colonies were counted. Clonogenic survival was expressed as the percentage of colony-forming units in treated cultures relative to the untreated controls.

Table 1 Comparison of protein expression levels of EGFR and ErbB2 and ZD1839- and C225-induced growth inhibition and clonogenic survival

Cell line	Relative EGFR expression ^a	Relative ErbB2 expression ^a	Growth inhibition			Clonogenic survival	
			ZD1839		C225	ZD1839	C225
			IC ₅₀ ^b	% (SD) ^c	% (SD) ^c	% (SD) ^d	% (SD) ^d
A431	15	4.0	0.1	77 (±3)	62 (±8)	55 (±7)	60 (±15)
A549	2.0	3.0	2.4	46 (±7)	49 (±11)	97 (±4)	97 (±15)
H1703	4.1	6.9	7.6	29 (±3)	35 (±11)	n.d. ^e	n.d.
SW1573	1.8	4.5	15	20 (±8)	19 (±10)	95 (±10)	108 (±14)
H460	1.5	3.1	24	25 (±5)	29 (±5)	105 (±5)	103 (±8)

^a Protein expression levels were analyzed by flow cytometry (see "Materials and Methods").

^b Drug concentrations (μM) responsible for 50% growth inhibition in MTT assay at 72 h, calculated with data of at least three independent experiments.

^c Growth inhibition induced by ZD1839 (1 μM) or C225 (5 μg/ml) represented as a percentage of untreated cells, analyzed by MTT assay at 72 h. Mean and SD of at least three independent experiments are shown.

^d Colony-forming units are expressed as a percentage of the respective untreated control. Mean and SD of one representative experiment done in triplicate are shown.

^e n.d., not determined.

Cell Cycle Analysis and Cell Death Measurement.

Cells were plated at a density of 1×10^5 cells/well in 6-well plates (Costar, Cambridge, MA). Twenty-four h later, the medium was replaced with medium containing the drug(s) as indicated. EGFR antagonists ZD1839 and C225 were used at concentrations of 1 μM and 5 μg/ml, respectively, and LY294002 and U0126 were used at concentrations of 30 and 10 μM, respectively. The broad-spectrum caspase inhibitor zVAD-fmk was added 1 h before treatment at a concentration of 50 μM when indicated. The cell cycle distribution of cells stained with PI was analyzed (19), and the extent of cell death was determined by measuring the sub-G₁ population. Apoptotic events were measured by annexin V-FITC and 7-AAD double staining according to the manufacturer's protocol (Nexins Research, Kattendijk, the Netherlands). All analyses were performed on a FACScalibur instrument using the CELLQuest or the ModFit 3.0 software packages (Becton Dickinson, Mount View, CA).

Caspase-3-like Enzyme Activity Assay. Caspase-3-like enzyme activity was assayed in cellular extracts using the ApoAlert caspase-3 kit (Clontech) according to the manufacturer's instructions. Fluorescence was detected using a Spectra Fluorimeter equipped with a 400 nm excitation filter and a 505 nm emission filter (Tecan). Relative caspase activity was expressed as the level of DEVD-AFC cleavage in the treated cells compared with the level in the untreated controls.

Detection of EGFR and ErbB2 Expression by Flow Cytometry. Cells (5×10^5) were harvested using trypsin and incubated for 1 h at 4°C with 1 μg of the anti-EGFR Mab C225 (Merck) or the anti-ErbB2 Ab-2 (Neomarkers, Fremont, CA). As a control for nonspecific binding, 1 μg of protein of human IgG1λ (Sigma) or mouse IgG1 (DAKO, Santa Barbara, CA) was used as isotype-matched nonbinding antibody for the EGFR and ErbB2, respectively. Subsequently, cells were washed twice with ice-cold PBS containing 0.5% BSA and incubated at 4°C in the dark for 1 h with FITC-conjugated goat antihuman or goat antimouse IgG antibody, diluted 1:50 in PBS/BSA. After two washing steps with ice-cold PBS/BSA, cells were resuspended in 0.5 ml of ice-cold PBS/BSA and analyzed on a FACScalibur flow cytometer using CELLQuest software (Becton Dickinson).

Relative expression levels were calculated as the ratio between the mean fluorescence intensity of cells stained with the specific antibodies and the mean fluorescence intensity of cells stained with the respective isotype-matched control antibody.

Western Blotting. Cell lysates were prepared in a buffer containing 20 mM HEPES/KOH (pH 7.4), 50 mM β-glycerophosphate, 50 mM KCl, 0.2 mM EDTA, 1% (w/v) Triton X-100, and 10% (w/v) glycerol. A protease inhibitor mixture (Roche, Almere, the Netherlands) and 1 mM NaVO₃ were freshly added to the lysis solution before each experiment. Protein concentrations were determined according to Bradford (20), using the Protein Assay Dye Reagent Solution (Bio-Rad) with BSA as a standard. Cell lysates were denatured in SDS, and equal amounts of protein were electrophoresed on 7–12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Subsequently, membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated overnight at 4°C with the appropriate primary antibodies. After incubation with horseradish peroxidase-conjugated goat antimouse or goat anti-rabbit antibodies for 1 h at room temperature, detection was performed using the enhanced chemiluminescence reagent (Amersham). The following antibodies were used: anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-Erk, anti-Erk, anti-phospho-GSK3β, and anti-phospho-p90^{RSK} (all from Cell Signaling Technology); and anti-PARP (Roche).

RESULTS

Differential Effects of Anti-EGFR Agents on Proliferation and Survival in A431 and NSCLC Cells. The growth of A431 cells is known to be potently inhibited by EGFR inhibitors (21–23). In this study, we have used MTT assays to determine the effect of ZD1839 and C225 on the growth of several NSCLC cell lines in comparison with the highly sensitive A431 cells. ZD1839 induced 50% growth inhibition in NSCLC cell lines at concentrations that were 24–240 times higher than the IC₅₀ for A431 cells (Table 1). At concentrations of ZD1839 greater than 1 μM, the antiproliferative effect of ZD1839 may be mediated

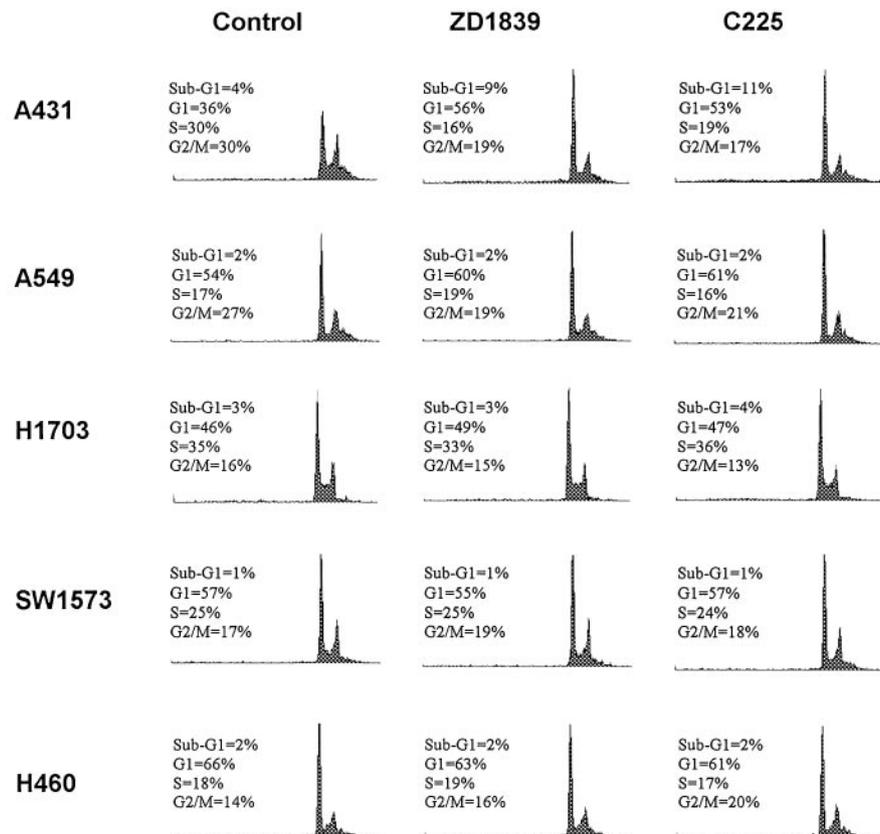


Fig. 1 Cell cycle analysis of cells treated with EGFR antagonists. A431 and NSCLC cells were stained with PI after a 72-h exposure to ZD1839 (1 μ M) or C225 (5 μ g/ml) and analyzed by flow cytometry. Percentages of the total cell population in the different phases of the cell cycle were determined and included. Representative results of at least three experiments are shown.

by actions additional to inhibition of EGFR (24). Therefore, the growth inhibition induced at 1 μ M ZD1839 compared with untreated control cells is also presented in Table 1, showing a growth inhibition of 77% in A431 cells at this concentration, whereas the growth inhibition ranged from not more than 20–30% in most lung cancer cells to 46% in the A549 cell line. Similarly, C225 induced a more pronounced growth inhibition of A431 cells than of the NSCLC cells (Table 1). However, compared with ZD1839, C225 was less potent in suppressing growth of A431 cells in the MTT assay (Table 1).

In the context of a cell population, growth inhibition may result from either decreased cell proliferation or decreased cell survival. To distinguish between these possibilities, we carried out clonogenic assays on cells exposed to ZD1839 or C225 for a limited period of time. Because the effect of ZD1839 is reversible upon removal (25), cells would be able to resume growth and give rise to a colony, unless their survival is compromised. ZD1839 did not reduce the clonogenic survival in any of the NSCLC cell lines, whereas treatment with ZD1839 resulted in a 50–60% reduction of A431-derived colonies (Table 1). Similar results were obtained with C225 in this assay (Table 1).

Effects of ZD1839 and C225 on Cell Cycle Progression.

To examine whether the inhibitory effects observed in growth assays reflect a delay or arrest of cells in the G_1 - G_0 phase, as shown previously (14, 26), cells were treated with ZD1839 (1 μ M) or C225 (5 μ g/ml) for 72 h, and the cell cycle progression

was evaluated after PI staining and fluorescence-activated cell-sorting analysis. An increase in the portion of cells in the G_1 - G_0 phase of the cell cycle by 17–20% in A431 cells and by 6–7% in A549 cells with a corresponding decrease in cells in S and G_2 -M phase was observed upon treatment with ZD1839 or C225 (Fig. 1), correlating with the antiproliferative effects observed in these cells (see Table 1). In contrast, in H1703, SW1573, and H460 cells, no change in cell cycle distribution was detected upon treatment with the EGFR inhibitors (Fig. 1), correlating with the limited antiproliferative effects observed in these cell lines at the concentrations used (see Table 1).

Interestingly, a significant ZD1839- and C225-dependent increase in the sub- G_1 cell population was detected in A431 cells, but not in any of the NSCLC cell lines (Fig. 1). Cells in sub- G_1 population may represent apoptotic cells, suggesting that the induction of apoptosis contributes to the cytotoxic effect of the EGFR inhibitors in A431 cells.

Cytotoxicity of ZD1839 in A431 Cells Is Blocked by Bcl-2 Overexpression and Is Partially Caspase Dependent. To investigate the involvement of apoptotic cell death in ZD1839-induced cytotoxicity in A431 cells, several known markers of apoptosis were evaluated. First, we examined the involvement of the antiapoptotic protein Bcl-2, which is able to stabilize the mitochondrial membrane, thereby preventing mitochondria-dependent caspase activation (27). It has been suggested that Bcl-2 family members have a role in apoptosis induced by

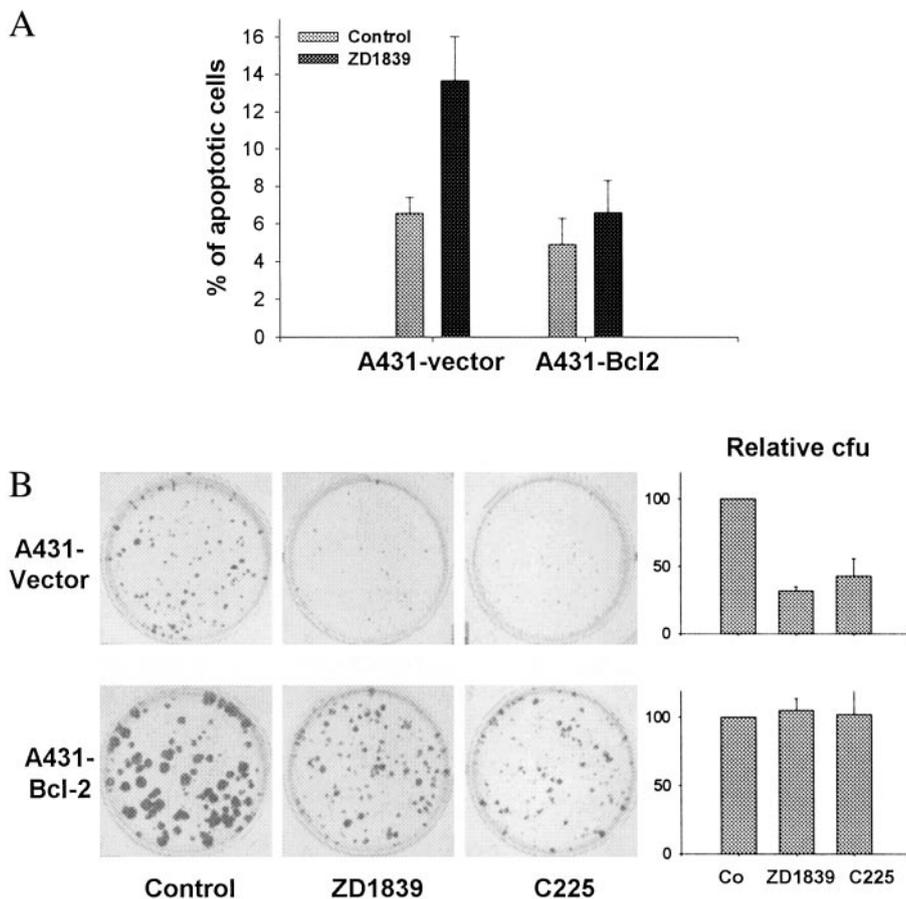


Fig. 2 Overexpression of Bcl-2 protects against ZD1839-induced PS externalization and results in clonogenic survival of A431 cells treated with ZD1839 or C225. A431 cells stably transfected with an empty retroviral vector (*A431-vector*) or a vector encoding Bcl-2 (*A431-Bcl-2*) were treated with ZD1839 (1 μ M) or C225 (5 μ g/ml) for 72 h. **A**, the percentage of apoptotic cells represents the fraction of cells that were positively stained with annexin V, measuring the amount of externalized PS, as analyzed by flow cytometry (see also Fig. 3). **B**, *left panels*, clonogenic survival of A431-Bcl-2 cells, but not of A431-vector cells. Pictures were taken from representative experiments. *Right panels*, colony-forming units (cfu) are expressed as a percentage of untreated cells and represent the mean \pm SD of at least three experiments.

EGFR-targeted agents (16, 28). To test the involvement of mitochondria in ZD1839-induced toxicity, we used retroviral transduction to generate an A431-derived cell line stably overexpressing Bcl-2. Overexpression of Bcl-2 prevented from the ZD1839-induced increase of externalized PS (Fig. 2A), a phospholipid that is normally confined to the inner leaflet of the plasma membrane and is externalized upon induction of apoptosis. To identify late apoptotic and necrotic cells, membrane integrity was investigated by staining with 7-AAD. In addition, Bcl-2 overexpression favored clonogenic survival of A431 cells treated with ZD1839 or C225 (Fig. 2B). Interestingly, cells overexpressing Bcl-2 formed colonies that were significantly larger in size than the control vector-transduced cells (Fig. 2B), further illustrating the prosurvival effect of Bcl-2.

As a marker for apoptotic cell death, the exposure of PS was analyzed by staining with annexin V-FITC. Upon treatment of A431 cells with ZD1839, a 2-fold increase of cells stained with annexin V-FITC was detected (Fig. 3A), which is comparable with the fraction of cells in sub- G_1 phase under these conditions. In ZD1839-treated NSCLC cells, no externalization of PS was observed (data not shown), corresponding with the lack of a cytotoxic effect in these cells. To examine the role of caspases in ZD1839-induced cell death, caspase-3-like activity was determined by measuring the cleavage of its fluorescent substrate, DEVD-AFC. Caspase-3-like activity increased 4.5

times in cells treated with ZD1839 compared with 11.7 times in cisplatin-treated cells (Fig. 3B), indicating that caspase-3 is activated to a lesser extent by ZD1839 than by cisplatin. As controls, ZD1839- and cisplatin-induced caspase-3-like activity was completely blocked when the broad spectrum caspase inhibitor zVAD-fmk was added during drug exposure (Fig. 3B), or when the specific caspase-3 inhibitor DEVD-acetyl-Asp-Glu-Val-Asp-aldehyde was added to the reaction mixture during the cleavage reaction (data not shown). Of note, zVAD-fmk decreased the proportion of cells with a hypodiploid DNA content induced by ZD1839 and completely prevented the appearance of the sub- G_1 population in cisplatin-treated cells (Fig. 3C). No PARP cleavage was detected in A431 cells treated with ZD1839, whereas in control cells treated with different concentrations of cisplatin, a dose-dependent increase in PARP cleavage was observed (Fig. 3D). The lack of PARP cleavage in ZD1839-treated cells could be due to the low amount of apoptosis that was induced, making it impossible to detect the cleaved fragment. In support of this, no PARP cleavage was detected in cells treated with 1 μ M cisplatin (Fig. 3D), a concentration that induces a sub- G_1 population (11%) similar to that resulting from ZD1839 treatment (data not shown). Taken together, these results demonstrate that the induction of caspase-dependent apoptosis contributes, at least in part, to the cytotoxicity induced by ZD1839 in A431 cells.

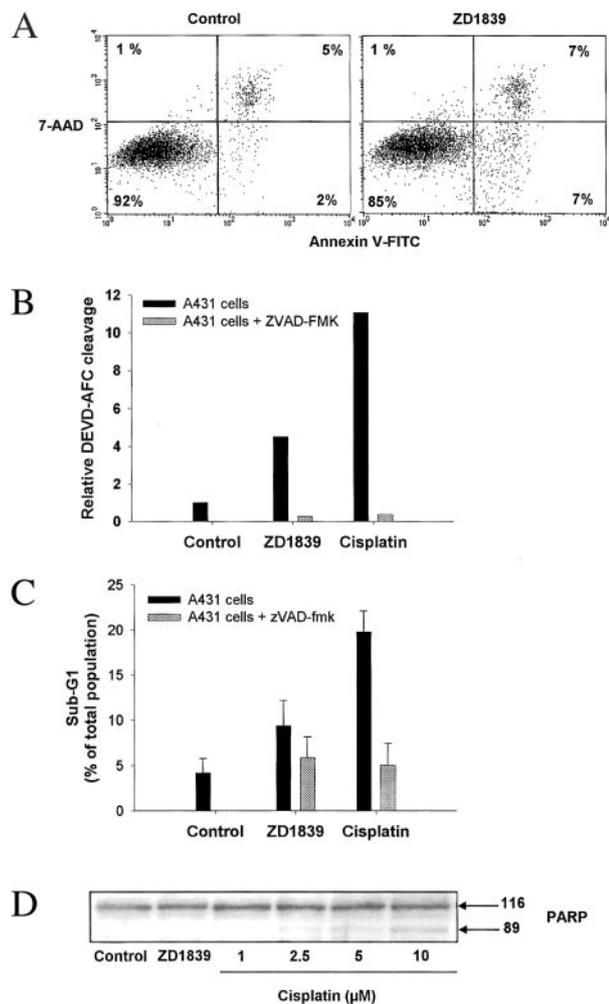


Fig. 3 Partial contribution of caspase-dependent apoptosis to ZD1839-induced cytotoxicity in A431 cells. *A*, cells treated with ZD1839 (1 μ M) for 72 h were double-stained with annexin V and 7-AAD and analyzed by flow cytometry. Gate settings distinguish between living (*bottom left*), necrotic (*top left*), early apoptotic (*bottom right*), and late apoptotic (*top right*) cells. *B*, DEVD-AFC cleavage was measured in treated and untreated cells in the presence or absence of caspase inhibitors. A representative experiment is shown. *C*, cells were treated with ZD1839 (1 μ M) for 72 h or cisplatin (10 μ M) for 48 h in the presence or absence of zVAD-fmk. Mean \pm SD values are shown from three experiments. *D*, Western blot analysis of PARP cleavage in A431 cells treated with ZD1839 (1 μ M) for 72 h or with different concentrations of cisplatin for 48 h.

Lack of Correlation between EGFR or ErbB2 Expression Levels and ZD1839 and C225 Toxicity in NSCLC Cells.

It is not yet known whether sensitivity to EGFR inhibitors depends on the amount of EGFR (23, 29, 30) and/or ErbB2 (22, 23, 26) expressed by cells. The NSCLC cell lines used in this study expressed moderate levels of EGFR when compared with the control A431 cell line, which expresses very high levels of EGFR (Table 1). Expression of ErbB2, on the other hand, was similar in all cell lines (Table 1). As reported previously (21–23), the highly EGFR-expressing A431 cells were extremely sensitive to the EGFR inhibitors, but neither EGFR nor ErbB2

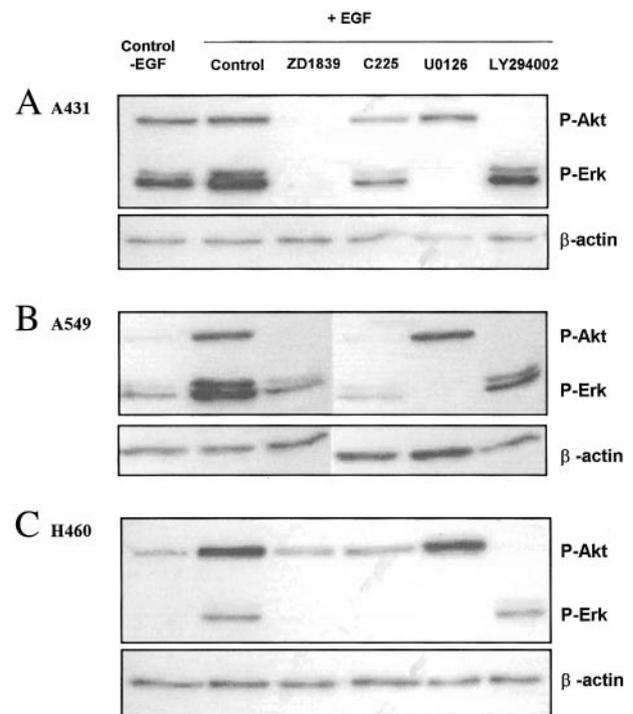


Fig. 4 Persistent activity of Erk or Akt in NSCLC cell lines, but not in A431 cells. *A–C*, A431, A549, and H460 cells were serum-starved overnight and treated with ZD1839 (1 μ M), C225 (5 μ g/ml), U0126 (10 μ M), or LY294002 (30 μ M) for 2 h before exposure to 10 ng/ml EGF for 5 min. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were immunostained for phosphorylated Erk and Akt. As a control for protein loading, membranes were stripped and reprobed with antibodies recognizing β -actin.

expression correlated with the growth-inhibitory effect of ZD1839 or C225 within the panel of NSCLC cell lines.

Effects of ZD1839 and C225 on Kinase Signaling Downstream of EGFR. EGFR signaling is transduced through two main kinase pathways, involving MEK/Erk and PI3K/Akt (31). Intrinsic activity of these pathways can potentially circumvent EGFR inhibition. Phosphorylation of Erk and Akt was analyzed to determine the activation status of the two pathways upon anti-EGFR treatment. The sensitive A431 cell line and two NSCLC cell lines (A549 and H460) were treated with the EGFR antagonists ZD1839 or C225, the MEK inhibitor U0126, or the PI3K inhibitor LY294002 before stimulation with EGF. Cell extracts were subsequently subjected to Western blot analysis. Nonstimulated A431 control cells displayed strong bands corresponding to phosphorylated Erk and Akt, and stimulation with EGF even resulted in a slight increase in phosphorylation (Fig. 4A, *Lanes 1* and *2*). Phosphorylation of Erk and Akt was abolished in ZD1839-treated A431 cells (Fig. 4A, *Lane 3*), which is in agreement with previous observations by others in these cells (21, 23). Compared with ZD1839, C225 only partially reduced phosphorylation of these downstream molecules in A431 cells (Fig. 4A, *Lane 4*). This may explain the smaller effect of the anti-EGFR antibody on growth, as shown earlier in the MTT assay, and suggests less effective EGFR kinase inhibition by C225 than by ZD1839 in A431 cells. As expected, the

MEK inhibitor (U0126) and the PI3K inhibitor (LY294002) specifically inhibited Erk or Akt phosphorylation, respectively (Fig. 4A, Lanes 5 and 6). Unlike A431 cells, phosphorylated Erk, but not Akt, was present in serum-deprived A549 cells (Fig. 4B, Lane 1), whereas the reverse was found in H460 cells, which had phosphorylated Akt and nonphosphorylated Erk (Fig. 4C, Lane 1), suggesting intrinsic activity of one of the kinase pathways in these cells. Incubation with EGF resulted in a significant increase of phosphorylated Erk and Akt in both cell lines (Fig. 4, B and C, Lane 2), demonstrating the functionality of the EGFR pathway in these cells. Treatment with ZD1839 or C225 resulted in the decrease of phosphorylation of Erk and Akt to the levels seen in the untreated controls, with activated Erk (in A549 cells) or Akt (in H460 cells) still detectable (Fig. 4, B and C, Lanes 3 and 4). However, in both lung cancer cell lines, treatment with U0126 or LY294002 completely abrogated Erk or Akt phosphorylation, respectively (Fig. 4, B and C, Lanes 5 and 6).

These results clearly indicate that the Erk or Akt kinase pathways are constitutively active and are not effectively blocked by EGFR antagonists in the NSCLC cell lines A549 and H460, respectively.

Dose-dependent Inhibition of EGF-induced Signaling via Erk and Akt Kinase Pathways by ZD1839. To further substantiate the latter findings, we extended our analysis to other NSCLC cell lines and treated them with different concentrations of ZD1839. In addition to Erk and Akt phosphorylation, the cell extracts were analyzed for phosphorylation of p90^{rsk} and GSK3 β , which are downstream substrates of Erk and Akt, respectively (32, 33). A dose-dependent decrease of Erk and Akt phosphorylation was observed in A431 cells, coinciding with decreased phosphorylation of p90^{rsk} and GSK3 β (Fig. 5A), indicating that both kinase pathways were blocked by ZD1839 in these cells. In A549 (Fig. 5B) and SW1573 cells (Fig. 5C), phosphorylation of Erk and p90^{rsk} was only partially inhibited, but a dose-dependent decline of Akt and GSK3 β phosphorylation was seen. In contrast, H460 cells showed dose-dependent reduction of Erk and p90^{rsk} phosphorylation but only a partial reduction of Akt and GSK3 β phosphorylation (Fig. 5D). In the H1703 cell line, no effects of ZD1839 were observed on the phosphorylation status of Erk, p90^{rsk}, Akt, and GSK3 β (Fig. 5E). The dose-dependent effect shows that inhibition of the Erk and Akt kinase pathways by ZD1839 is the consequence of EGFR blocking. Furthermore, these data confirm that at least one of the kinase pathways involving Erk and Akt is persistently active in the presence of ZD1839 in the NSCLC cells, whereas both pathways are effectively blocked in A431 cells.

Simultaneous Inhibition of the MEK and PI3K Pathways Results in Effective Inhibition of Growth and Apoptosis. The above-mentioned results suggest that persistent activity of the MEK and/or PI3K pathway contributes to the resistance of NSCLC cells to EGFR antagonists. To independently investigate the role of MEK and PI3K in proliferation, we determined the effect of U0126 and LY294002 on the growth of A431, A549, and H460 cells. As single agents, both inhibitors induced a dose-dependent growth inhibition that was similar in all cell lines in a 72-h MTT assay (Fig. 6, A–C), demonstrating a role of both kinase pathways in the proliferation of these cells. When cells were treated with a combination of U0126 and

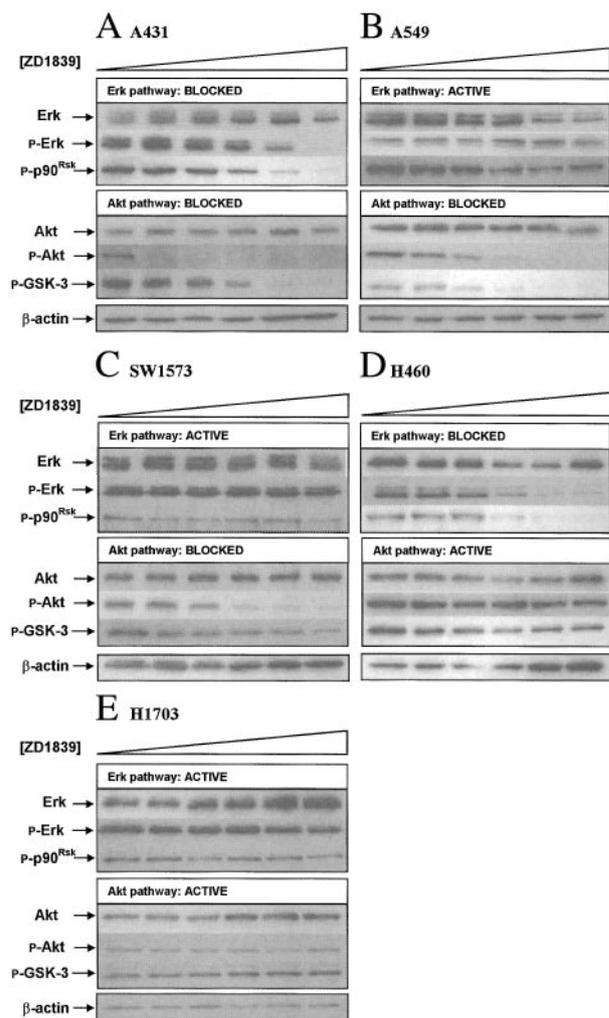


Fig. 5 Dose-dependent inhibition of Erk and Akt kinase pathways by ZD1839. Serum-starved A431 cells (A) and NSCLC cells (B–E) were treated with different concentrations of ZD1839 (0, 0.05, 0.1, 0.25, 0.5, and 1 μ M) for 2 h before stimulation with 10 ng/ml EGF for 5 min. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were immunostained for phosphorylated and total Erk and Akt, phosphorylated p90^{rsk}, and phosphorylated GSK3 β . As a control for equal protein loading, membranes were stripped and reprobed with antibodies recognizing β -actin. *BLOCKED*, effective inhibition of the Erk or Akt pathway by ZD1839; *ACTIVE*, persistent activity of the Erk or Akt pathway after treatment with ZD1839.

LY294002, the effect on growth was at least additive in all cell lines (Fig. 6, A–C), suggesting an independent role of the MEK and PI3K pathway in proliferation. When compared with U0126 and LY294002 as single agents or in combination, ZD1839 was more effective in inhibiting the growth of A431 cells (Fig. 6A), indicating that ZD1839 is more potent in inhibiting its target than U0126 or LY294002 and that A431 cells are largely dependent on EGFR activity for their growth. In contrast, treatment of NSCLC cells with U0126 and/or LY294002 produced a more pronounced inhibition of cell growth than treatment with ZD1839 (Fig. 6, B and C). This can be explained by the fact that

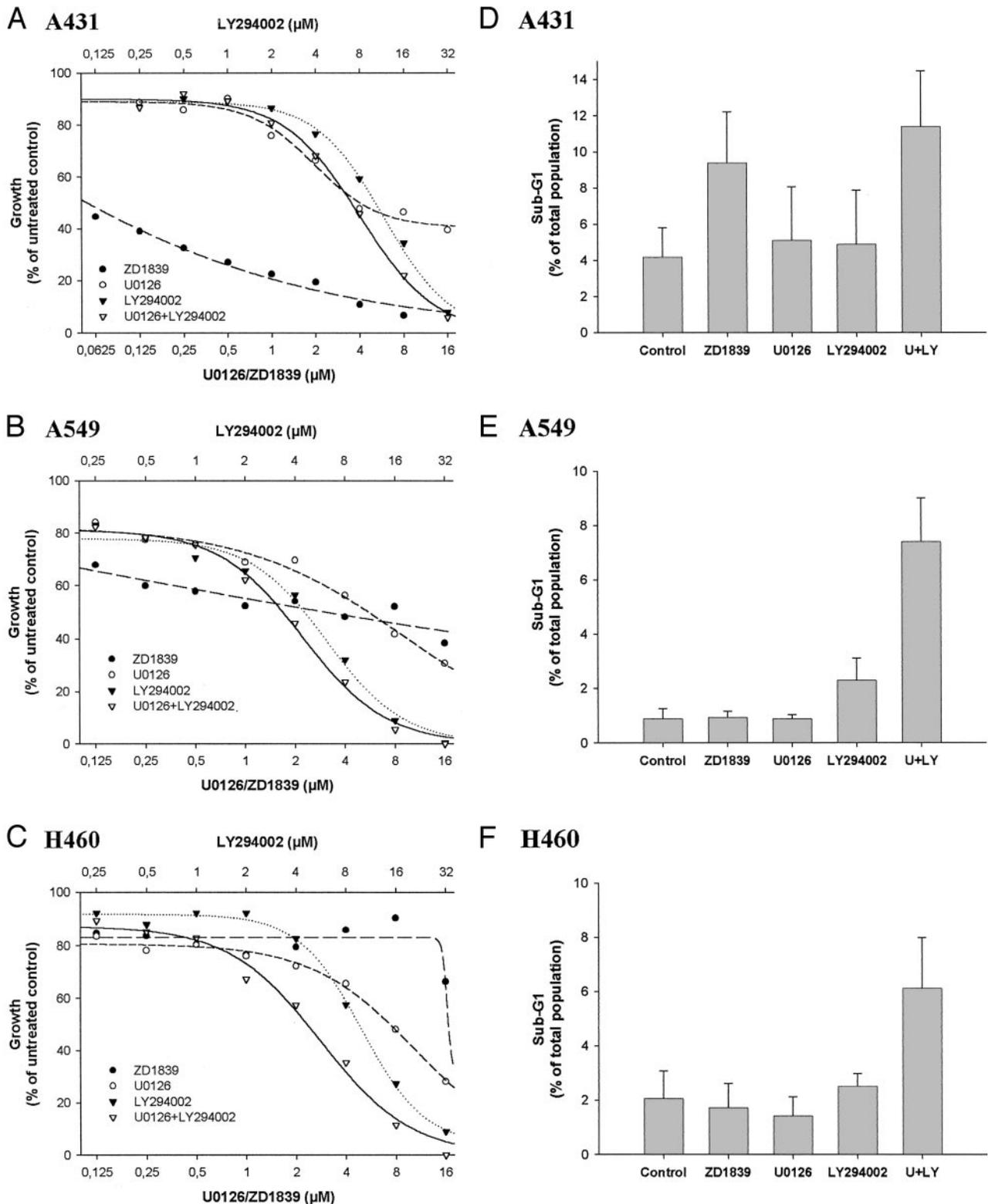


Fig. 6 Effective growth inhibition and induction of apoptosis in cells with inhibited MEK and PI3K. A–C, growth curves of A431, A549, and H460 cells treated with different concentrations of ZD1839, U0126, LY294002, or a combination of U0126 and LY294002 with a ratio of 1:2. Growth was represented as a percentage of untreated cells, analyzed by MTT assay at 72 h. The plots were fitted using Sigma plot software (four-parameter logistic curve). Similar results were obtained in at least three independent experiments. D–E, analysis of the sub-G₁ population induced by ZD1839 (1 μM), U0126 (10 μM), LY294002 (30 μM), or a combination of U0126 and LY294002. A431, A549, and H460 cells were treated with the kinase inhibitors for 72 h, subsequently stained with PI, and analyzed by flow cytometry.

ZD1839 specifically blocks EGFR-dependent signaling, whereas activation of downstream pathways in the presence of EGFR inhibitors is still possible by enhanced activity of proteins such as Ras or PI3K (34, 35) or via other receptors that may be activated by growth factors or hormones in the serum (22). U0126 and LY294002 directly inhibit the MEK or PI3K pathway, thereby blocking EGFR-dependent and -independent signaling and inducing stronger antiproliferative effects. In addition, despite the selectivity of the MEK and PI3K inhibitors, we cannot rule out their potential effects on other targets involved in proliferation, particularly at higher concentrations (36).

In addition, we used U0126 and LY294002 to determine the role of the MEK and PI3K pathways on apoptosis in A431, A549, and H460 cells. As shown earlier, ZD1839 induced a 2–3-fold increase of A431 cells with hypodiploid DNA, whereas no effect was observed in the A549 or H460 cell line (Fig. 1; Fig. 6, D–F). Neither U0126 nor LY294002 induced changes in the sub-G₁ population as a single agent in any of the cell lines, whereas the combination of both agents induced an increase in the sub-G₁ population in A431 as well as the lung cancer cells to a level that was similar to the sub-G₁ population induced by ZD1839 in A431 cells (Fig. 6, D–F). These data indicate that the inhibition of the MEK and the PI3K pathway in A431 cells by ZD1839 can explain the cell death induced in these cells and support the view that the persistent activity of at least one of these pathways may protect the NSCLC cells from ZD1839-induced apoptosis.

DISCUSSION

In this study, we show that treatment of NSCLC cell lines with EGFR inhibitors induces differential antiproliferative effects, although the effect on growth is limited when compared with the control A431 cell line. In the highly sensitive A431 cell line and, to a lesser extent, also in A549 cells, the growth-inhibitory effect of the EGFR antagonists correlates with an arrest of cells in the G₁-G₀ phase of the cell cycle, whereas the cell cycle is unaffected in the more resistant lung cancer cell lines (Fig. 1). These results are consistent with earlier reports, showing that antiproliferative effects of ZD1839 or C225 correlate with a G₁-G₀-phase cell cycle arrest in cell lines derived from breast and head and neck tumors (14, 26).

We demonstrate that treatment with EGFR inhibitors induces a modest apoptotic response in A431 cells but does not affect the survival of NSCLC cells (Table 1; Fig. 3). Comparable amounts of apoptosis have been reported for several other cell lines treated with ZD1839 or C225 (13, 14), whereas effective induction of apoptosis by EGFR-targeted agents has been reported in only a few cell lines (16, 17). In line with an earlier report demonstrating that ZD1839 induces apoptosis by activating the proapoptotic Bcl-2 family member BAD in mammary cells (16), we found that overexpression of Bcl-2 prevents ZD1839- and C225-induced cell death in A431 cells. We further show that caspases contribute, at least in part, to the cytotoxicity of ZD1839 in A431 cells.

We investigated molecular differences that may underlie the variable sensitivity of A431 and NSCLC cells to anti-EGFR agents. It has been reported that cells with high expression of EGFR (21, 23) or ErbB2 (22, 23, 26) are particularly sensitive

to ZD1839, suggesting that abundant expression of EGFR or ErbB2 is required to modulate sensitivity. However, Moasser *et al.* (23) also demonstrated that high endogenous EGFR expression *per se* does not determine sensitivity to EGFR inhibitors. Because EGFR and ErbB2 are part of a large signaling network (4), a number of factors rather than expression levels of the receptors alone may determine the sensitivity of a cell to EGFR inhibitors. In support of this view, we found that the sensitivity to the anti-EGFR agents within the panel of lung cancer cells differed significantly, although all expressed similar, moderate protein levels of EGFR compared with the highly EGFR-expressing A431 cell line, whereas the ErbB2 expression levels were similar in all cell lines, including A431. This indicates that neither EGFR nor ErbB2 expression levels correlate with sensitivity to EGFR inhibitors in NSCLC cells.

Next, we determined the activation status of the MEK and PI3K pathways, two major intracellular signaling pathways activated by the EGFR. Sensitivity to EGFR inhibitors was found to correlate with persistent activity of these pathways in the presence of ZD1839 in the panel of NSCLC cell lines, whereas they are effectively blocked in the A431 cell line (Fig. 5). In line with this, Brognard *et al.* (37, 38) recently showed that Erk and Akt were constitutively active in the majority of NSCLC cell lines deprived of serum. Moreover, our results showing more effective antiproliferative effects when both pathways are blocked indicate that the MEK and PI3K pathways may independently contribute to the proliferation of these cells (Fig. 6, B and C). These data are in line with previous evidence showing that intrinsically active Erk (30) and Akt (23) correlate with reduced antiproliferative activity of ZD1839 in several epithelial tumor cell lines, whereas the growth-inhibitory effect of the HER2-targeted Mab Herceptin is prevented in breast cancer cells ectopically overexpressing an active mutant of Akt (39).

In addition to their contribution to cell proliferation, persistently active Erk and Akt pathways may protect cells from apoptosis induced by EGFR-targeted agents. Earlier reports demonstrated that cells transfected with active mutants of members of the MEK/Erk or PI3K/Akt pathway bypassed apoptosis induced by ZD1839 (16) or Herceptin (39). We found that chemical inhibition of either MEK or PI3K did not generate apoptosis of A431 or NSCLC cells (Fig. 6, C–F), which was also recently shown in A549 and other NSCLC cell lines (37, 38). In contrast, disabling the MEK and the PI3K pathway resulted in the induction of a modest apoptotic response in the A431 and lung cancer cell lines, similar to the amount of apoptosis induced by ZD1839 in A431 cells (Fig. 6, C–F). These data further support the idea that persistently active MEK and PI3K pathways, which are present in the NSCLC cell lines, account for the unaffected survival of these cells in the presence of anti-EGFR agents.

The limited effect observed *in vitro* contrasts with some *in vivo* studies reporting complete regressions of A431-derived tumors in mice treated with ZD1839 or C225 and 70–80% growth inhibition of A549-derived tumors in ZD1839-treated mice (15, 24, 40). Mechanisms that are only active *in vivo* may explain the more effective antitumor activity *in vivo* compared with the limited antiproliferative effects *in vitro*, such as those observed in A549 cells. First, C225 and ZD1839 can inhibit angiogenesis, which has been proposed to be the result of

reduced secretion of angiogenesis factors (41–45), whereas anti-EGFR agents can also directly inhibit the growth and cell-cell interaction of endothelial cells (24, 46). Second, C225 was shown to inhibit metastasis of bladder carcinoma xenografts (44), demonstrating the implication of EGFR signaling in cell migration and invasion (47, 48).

The results presented here, showing that constitutively active Erk and Akt could contribute to resistance to anti-EGFR treatment, may have important clinical relevance. To select patients that benefit from anti-EGFR therapy, it may be important to identify tumors that do not carry intrinsically active Erk or Akt. Immunohistochemical analysis of tumors for activated Erk and Akt may predict response to ZD1839 and C225 and will give more insight if constitutively activated Erk and/or Akt correlate with higher resistance to anti-EGFR treatment in patients. In fact, activated Erk has been correlated with EGFR activity in tumors (22, 49) and is down-regulated in the skin from cancer patients treated with ZD1839 (50), suggesting that activated Erk can be used as a marker for EGFR activity *in vivo*. On the other hand, anti-EGFR treatment in combination with specific inhibitors targeting kinase pathways via MEK or PI3K, some of which are now being tested in preclinical and clinical studies (51), might result in additional antitumor effect in some types of NSCLC.

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Clinical Cancer Research

Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small Cell Lung Cancer Cells: Limited Antiproliferative Effects and Absence of Apoptosis Associated with Persistent Activity of Extracellular Signal-regulated Kinase or Akt Kinase Pathways

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