# Modulation of survival pathways in ovarian carcinoma cell lines resistant to platinum compounds

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## Abstract

**Because cytotoxic stress elicits various signaling pathways that may be implicated in cell survival or cell death, their alterations may have relevance in the development of platinum-resistant phenotype. Thus, in the present study, we investigated cell response to the epidermal growth factor receptor (EGFR) inhibitor gefitinib of ovarian carcinoma cell lines, including cells selected for resistance to cisplatin (IGROV-1/Pt1) and oxaliplatin (IGROV-1/OHP). Resistant sublines exhibited a marked decrease in sensitivity to gefitinib and resistance to apoptosis. Gefitinib was capable of inhibiting the phosphorylation of EGFR in all the studied cell lines. The Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) kinases, which act downstream of EGFR, were constitutively active in the three cell lines, but phospho-ERK1/2 levels were increased in the two resistant sublines. This feature was associated with reduced sensitivity to the MEK1/2 inhibitor U0126. Pretreatment of resistant cells with U0126 resulted in restoration of sensitivity to gefitinib. Gefitinib was more effective in inhibiting ERK1/2 and Akt phosphorylation in IGROV-1 cells than in IGROV-1/OHP and IGROV-1/Pt1 cells. Phospho-p38 was up-regulated in the resistant sublines, indicating the concomitant activation of distinct mitogen-activated protein kinases. The up-regulation of phospho-p38 was associated with a peculiar localization of EGFR, which, in resistant sublines, was mainly internalized. In conclusion, our results indicate that the development of resistance to platinum drugs is associated with multiple alterations including deregulation of survival pathways activated by EGFR resulting in a reduced cellular response to gefitinib. [Mol Cancer Ther 2008;7(3):679–87]**

## Introduction

The occurrence of resistance to treatment represents a major limitation to the efficacy of platinum-based therapies in different malignancies, including ovarian carcinoma (1). Cellular resistance to cisplatin (cDDP) is a complex phenomenon involving multiple alterations such as increased defense mechanisms, augmentation of DNA repair, and inhibition of apoptosis (2). Moreover, activation of survival pathways is a common feature of cancer cells and could influence the cellular response after drug treatment (3). In particular, alterations in signaling pathways mediated by the ErbB family of receptors have been documented in ovarian carcinoma cells (4). Available evidence supports that the epidermal growth factor receptor (EGFR) expression is implicated in the progression of disease (5). Patients with alterations in tyrosine kinase receptors tend to have a more aggressive disease and poor prognosis (6). Thus, the EGFR pathway may affect cellular response to cDDP, because the drug is known to modulate the signaling occurring through activation of EGFR or EGFR-mediated activation of downstream events (7). In this context, the mitogen-activated protein kinase (MAPK) cascades, which are involved in both growth factor and stress response signaling, play a complex and controversial role in determining the ultimate fate of the cells depending on cell type and molecular background (8, 9). In ovarian cancer, the extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK pathway has been implicated in signaling of proliferation, differentiation, and survival (10).

The activation of survival pathways, involving the MAPK or phosphatidylinositol 3-kinase/Akt cascade, in response to cytotoxic stresses appears to be largely mediated by growth factor receptors (in particular, EGFR). Among targeted agents, the interest for gefitinib (ZD1839, Iressa), a selective inhibitor of EGFR, has emerged due to preclinical studies documenting marked antitumor activity as well as oral bioavailability and tolerability (11, 12). The emergence of target-specific agents in cancer therapies represents a relevant aspect for the treatment of tumors and is expected to allow the definition of novel therapeutic strategies for tumors resistant to conventional cytotoxic agents (13). Because EGFR has been implicated in the progression of ovarian carcinoma and likely in the development of drug resistance (5), and impairment of pathways mediated by the ErbB family of receptors have been reported in ovarian carcinoma cells (4), the aim of the

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present study was to examine cell response to gefitinib in ovarian carcinoma cell lines expressing physiologic levels of EGFR, including cells resistant to platinum compounds with particular reference to survival pathways. We found that cells selected for resistance to cDDP (IGROV-1/Pt1) and oxaliplatin (IGROV-1/OHP) displayed reduced sensitivity to gefitinib and concomitant activation of distinct MAPKs. Thus, the development of resistance to platinum drugs may result in significant changes in survival pathways affecting cell response to gefitinib.

## Materials and Methods

### Cell Culture and Drugs

The human ovarian carcinoma IGROV-1 cell line and the cDDP- and OHP-resistant sublines, IGROV-1/Pt1 and IGROV-1/OHP, were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% FCS (Invitrogen). The IGROV-1/Pt1 variant was generated as described (14). IGROV-1/OHP cells were developed by exposure of the parental cell line to increasing concentrations of OHP. Resistance was stable for at least 6 months when cells were grown in the absence of selecting agent. cDDP (Platinex; Bristol-Myers Squibb) was diluted in saline. OHP (Eloxatin; Sanofi-Synthelabo) was reconstituted in water and diluted in saline. Gefitinib, kindly provided by AstraZeneca, and the MEK1/2 inhibitor U0126 (Sigma) were dissolved in DMSO. Both drugs were diluted in medium before use. The concentration of DMSO in culture medium of treated cells never exceeded 0.5%.

### Growth Inhibition Assay

Cell sensitivity was assessed by growth inhibition assay based on cell counting (15). Twenty-four hours after seeding, cells were exposed to cDDP or OHP for 1 h and incubated for 72 h in drug-free medium. Exposure to gefitinib or U0126 was for 72 h. Culture medium was then removed and adherent cells were harvested using trypsin and counted with a cell counter (Coulter Electronics). The total number of cells remaining attached to the plastics at the end of the experiment was used to calculate the percent of growth inhibition. $IC_{50}$ is defined as the concentration causing a 50% inhibition of cell growth compared with control. In combination experiments, 24 h after seeding, cells were treated with the MAPK inhibitor U0126 for 4 h and then coincubated with U0126 and gefitinib for 72 h. At the end of treatment, cells were counted as detailed above.

### DNA Platination

DNA platination was measured as described previously (16). Cells were exposed to cDDP for 1 h. DNA was extracted according to standard procedures involving lysis in the presence of 1 mg/mL proteinase K overnight at 37°C. DNA was then isolated following phenol extraction, ethanol precipitation, RNase treatment, and reprecipitation and finally was dissolved in 10 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L EDTA. DNA content was determined spectrophotometrically and platinum content was measured by inductively coupled plasma-mass spectrometry (17).

### Mutational Analysis of EGFR, PTEN, and p53

Total RNA was isolated with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (1 μg) was converted to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). The cDNAs were used to amplify the kinase domain of EGFR (from 2,095 to 2,942 nucleotides of the coding sequence) and PTEN (from 841 to 1,080 nucleotides of the coding sequence) in the nucleotide sequence containing the reported mutation (Het c.955_958delACTT; ref. 18). The mutational analysis of p53 was done using genomic DNA as template. The primers used were as follows: for EGFR kinase domain, forward 5′-cccaaccaagctctcttgag-3′ and reverse 5′-atgacaaggtagcgctggggg-3′; for PTEN, forward 5′-ccaggaccagaggaaacct-3′ and reverse 5′-gctagcctctggatttgacg-3′; and for p53, forward 5′-ttcctcttcctgcagtactc-3′ and reverse 5′-accctgggcaaccagccctgt-3′ (exon 5), 5′-acagggctggttgcccagggt-3′ and 5′-agttgcaaaccagacctcag-3′ (exon 6), 5′-gtgttgtctcctaggttggc-3′ and 5′-gtcagaggcaagcagaggct-3′ (exon 7), and 5′-tatcctgagtagtggtaatc-3′ and 5′-aagtgaatctgaggcataac-3′ (exon 8). PCR conditions were as follows: initial denaturation at 95°C for 3 min followed by 30 cycles at 95°C for 1 min, 50°C for EGFR/55°C for PTEN/58°C for p53 for 1 min, 68°C for 2 min (EGFR and PTEN)/72°C for 1 min (p53), and finally 68°C for 5 min. The amplified products were automatically sequenced using the same primers and the ABIPRISM Dye Terminator cycle 6 sequencing ready reaction kit (Perkin-Elmer).

### Apoptosis Measurement

Apoptosis was measured by Annexin V binding assay. The Annexin V binding assay (Bender MedSystem) was done as described (19). Cells were seeded in 24-well plates and exposed to the drugs for the indicated times and then harvested. Floating cells were collected from the medium by centrifugation, whereas nonenzymatic cell dissociation solution (Sigma) was used for adherent cells. Total cells were processed for Annexin V binding assay following the manufacturer's instructions. Fluorescence was measured by flow cytometry using a FACScan (Becton-Dickinson) and data were analyzed with a specific software (Cell Quest; Becton-Dickinson).

### Western Blot Analysis

Western blot analysis of total cell lysates was done as described previously (20). Briefly, adherent and floating cells were collected and rinsed with cold PBS plus 0.1 mmol/L sodium orthovanadate. For cell fractionation experiments, the nuclear extract kit (Active Motif, Vinci Biochem) was employed following the manufacturer's instructions. After SDS-PAGE, cell extracts were transferred to nitrocellulose membranes and filters were blocked with in 1% bovine serum albumin, 3% (w/v) ovalbumin in TBS-T [25 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 0.1% Tween 20] and incubated with primary antibodies overnight at 4°C. Enhanced chemiluminescence (Amersham Pharmacia Biotech) was used as a detection system. The used primary antibodies were against phospho-p44/42 MAPK $Thr^{202}/Thr^{204}$, phospho-Akt $Ser^{473}$, cleaved caspase-3 $Asp^{175}$, phospho-p38 $Thr^{180}/Tyr^{182}$, c-Jun $NH_2$-terminal

**Table 1. Sensitivity of IGROV-1, IGROV-1/OHP, and IGROV-1/Pt1 cells to platinum compounds, to the EGFR inhibitor gefitinib, and to the MEK1/2 inhibitor U0126**

| Drug | $IC_{50}$ (μmol/L) | | |
|---|---|---|---|
| | IGROV-1 | IGROV-1/OHP | IGROV-1/Pt1 |
| cDDP (1 h) | 7.3 ± 1.6 | 50.96 ± 9.0 (7)* | 94.1 ± 14.0 (13)* |
| OHP (1 h) | 12.7 ± 3.1 | 928.5 ± 78.0 (73)* | 171.5 ± 0.2 (13)* |
| Gefitinib (72 h) | 0.18 ± 0.05 | 3.34 ± 0.9 (19)* | 9.12 ± 2.0 (51)* |
| U0126 (72 h) | 0.025 ± 0.001 | 5.95 ± 3.1 (238)* | 8.4 ± 1.8 (336)* |

NOTE: Cell sensitivity to drug was assessed by growth inhibition assay. Twenty-four hours after seeding, cells were exposed for 1 h to cDDP or OHP and were counted 72 h after treatment. For gefitinib and U0126, treatment was for 72 h and cells were counted at the end of treatment. Mean ± SD of three to six independent experiments.

*Degree of resistance, ratio between the $IC_{50}$ value of resistant and parental cells.

kinase (JNK; Cell Signaling Technology), MAPK1/2 (ERK1/2; Upstate Biotechnology), phospho-EGFR (pY1086; Biosource), protein kinase Bα/Akt, topoisomerase I (BD Transduction Laboratories), caspase-8 (BD PharMingen), MKP-1, MKP-3, phospho-JNK $Thr^{183}/Tyr^{185}$, p38 (Santa Cruz Biotechnology), survivin and XIAP (Abcam), actin, and β-tubulin (Sigma). All experiments were done at least three times.

### Immunofluorescence Assay

Cells were plated on coverslips and 24 h after seeding were treated with cDDP for 1 h. After treatment, cells were washed with PBS, fixed in 3% paraformaldehyde, permeabilized with 100% methanol at -30°C, and incubated with an anti-EGFR antibody (Upstate Biotechnology) overnight at 4°C. After washing with PBS, cells were incubated with a goat anti-mouse IgG Alexa Fluor 488–conjugated antibody (Molecular Probes/Invitrogen) for 1 h at room temperature and then stained with Hoechst 33342. Coverslips were mounted in Mowiol and observed using an upright fluorescence microscope (Leica DMRB; Leica Microsystems) equipped with a CCD and analyzed with the IAS2000 software (Delta Sistemi).

## Results

### Phenotype of Platinum-Resistant Cells

The platinum-resistant sublines, IGROV-1/OHP and IGROV-1/Pt1, were generated by chronic exposure to OHP and cDDP, respectively, and were both resistant to cDDP and OHP (Table 1; ref. 14). Resistance to platinum drugs was associated with increased damage tolerance (Fig. 1A). In fact, when cells were exposed for 1 h to 300 μmol/L cDDP, a higher amount of DNA-bound platinum was observed in the resistant sublines, with IGROV-1/Pt1 cells exhibiting the highest level. The found differences were maintained also when cells were incubated in drug-free medium for 5 or 24 h.

Resistance to platinum drugs was associated with p53 mutation in both resistant variants. Indeed, sequence analysis of genomic DNA of IGROV-1/OHP cells indicated the occurrence of a missense mutation affecting exon 6 (TAT→TGT, Tyr→Cys, amino acids 205). The presence of mutant p53 in IGROV-1/Pt1 has been described previously (14).

Apoptosis was measured in the three cell lines exposed for 1 h to 200 μmol/L OHP or 30 μmol/L cDDP and harvested 48 h later. In both resistant variants, reduced levels of apoptosis after exposure to platinum drugs were documented compared with parental cells (Table 2).

### Cell Sensitivity of Ovarian Carcinoma Cell Lines to Gefitinib

Cell sensitivity to gefitinib was assessed using growth inhibition assay after 72-h drug exposure. The platinum-resistant sublines displayed decreased sensitivity to gefitinib, with IGROV-1/Pt1 cells being the least sensitive (Table 1). The reduced antiproliferative effect of gefitinib in the platinum-resistant sublines was associated with a decreased induction of apoptosis as shown by Annexin V binding assay after 72-h exposure to gefitinib and by lower activation of caspases (Fig. 1B; Table 2). Indeed, caspase-3 and caspase-8 activation was already evident in parental IGROV-1 cells after 48-h exposure to gefitinib. A marginal activation of caspase-3 was found in IGROV-1/OHP cells but not in IGROV-1/Pt1 cells after 72-h exposure to gefitinib, in keeping with the relative resistance.

Previous evidence has suggested that the apoptotic effect of gefitinib in IGROV-1 cells involves down-regulation of survivin levels (21). We therefore analyzed modulation of survivin and XIAP by gefitinib in sensitive and resistant cell lines (Fig. 1C). A different cell response to gefitinib treatment was observed. In fact, a 24-h exposure to gefitinib resulted in down-regulation of survivin and, to a lesser extent, of XIAP in parental cells. In contrast, down-regulation of survivin was observed in IGROV-1/OHP cells, without modulation of XIAP. Finally, no changes of survivin or XIAP were detected in the most resistant subline, IGROV-1/Pt1.

### EGFR Gene Status and Modulation of Phosphorylation

Because mutations of the EGFR kinase domain have been implicated in sensitivity/resistance to treatment with gefitinib (22–24), we analyzed the sequence of the receptor kinase domain in the parental and resistant cell lines. However, no mutations were found. Thus, to determine whether the effects of gefitinib on the cell systems were related to different target inhibition, cells were exposed to 10 μmol/L gefitinib and the phosphorylation status of EGFR was examined. Western blot analysis using a phosphospecific antibody indicated a reduction of EGFR phosphorylation at 24 h in the three cell lines (Fig. 2A). A slight increase in baseline levels of phospho-EGFR was observed in resistant cells, in keeping with total EGFR content. Under our experimental conditions, gefitinib displayed an effect on Akt and ERK1/2 activities in IGROV-1 and IGROV-1/OHP cells, whereas such an inhibition was not evident in IGROV-1/Pt1 cells (Fig. 2A).

### Activation of Survival Pathways

In an attempt to investigate possible alterations in the signaling pathways activated by EGFR, we examined the expression/activation of MAPKs and Akt, which are

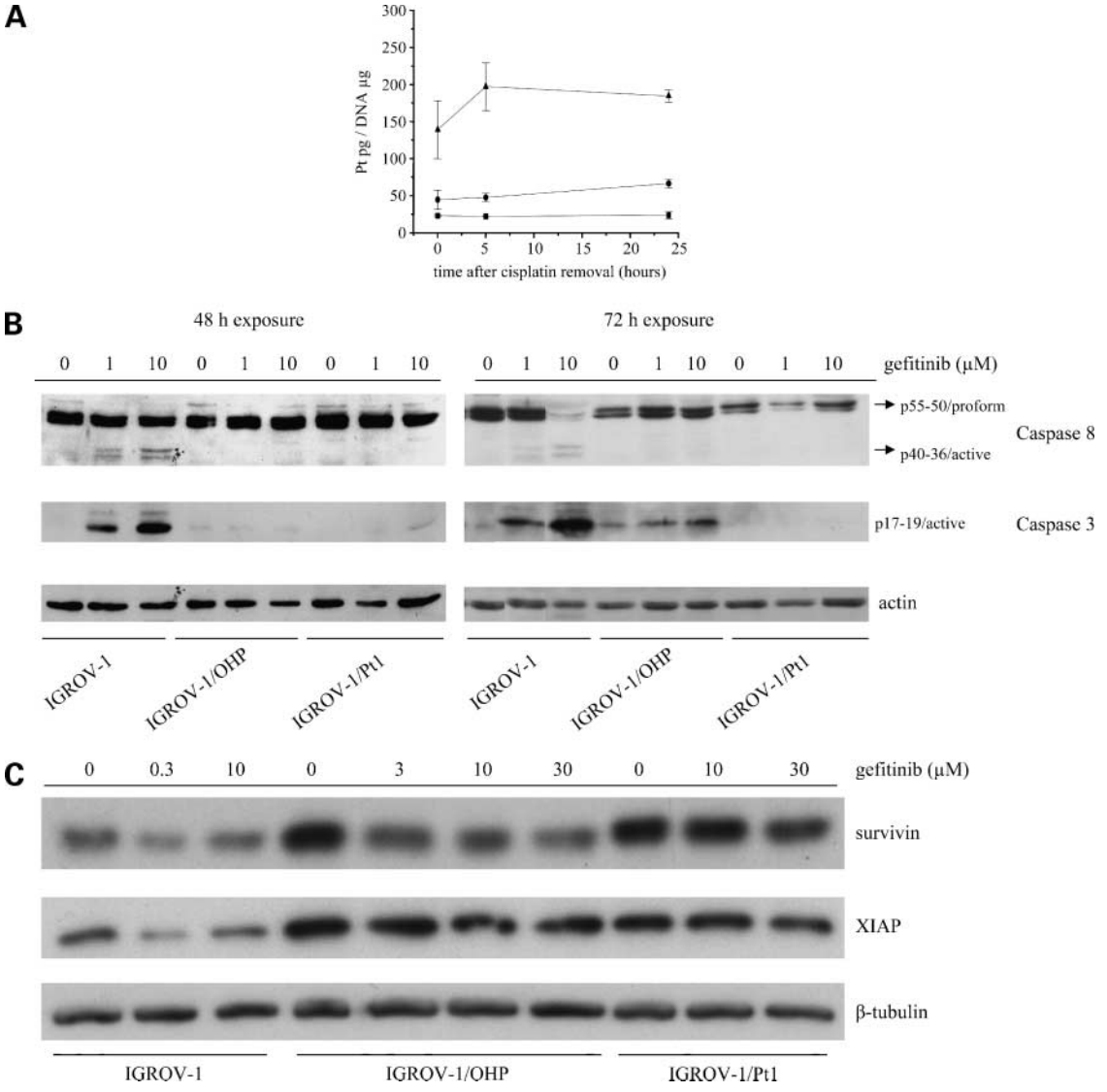


**Figure 1.** **A,** time course of DNA platination after 1-h cDDP exposure (300 µmol/L) and at two different times after cDDP removal (5 and 24 h). —■—, IGROV-1; —●—, IGROV-1/OHP; —▲—, IGROV-1/Pt1. **B,** Western blot analysis of caspase-8 and caspase-3 activation after 48- and 72-h exposure to equimolar concentration of gefitinib. Control loading is shown by actin. **C,** Western blot analysis of survivin and XIAP after gefitinib exposure in ovarian carcinoma cell lines. Cells were exposed to concentration corresponding to $IC_{50}$ (0.3 µmol/L for IGROV-1, 3 µmol/L for IGROV-1/OHP, and 10 µmol/L for IGROV-1/Pt1) and $IC_{80}$ (10 µmol/L for IGROV-1 and 30 µmol/L for IGROV-1/OHP and IGROV-1/Pt1) for 24 h and then harvested for Western blot analysis.

implicated in the signal transduction. In principle, intrinsic activity of the kinases could bypass gefitinib-mediated EGFR inhibition. Expression and activation of ERK1/2 and Akt were analyzed in cells grown in standard conditions (10% FCS) or deprived of serum for 24 h. Although the expression level of the kinases was similar in parental and resistant cells under both conditions, the resistant sublines showed a higher basal level of ERK phosphorylation compared with the parental cells (Fig. 2B). Increased expression of activated ERK1/2 in the resistant sublines was associated with resistance to the MEK1/2 inhibitor U0126 (Table 1). The observed resistance reflected reduced susceptibility to apoptosis (Table 2). Because a heterozygous mutation of PTEN, a phosphatase that negatively regulates Akt, has been described in IGROV-1 cells (18), we examined the presence of this mutation in resistant cells. Constitutive activation of Akt was associated with heterozygous mutation of PTEN (Het c.955_958delACTT) in all cell lines.

In comparison with the parental cells, the resistant sublines revealed increased phospho-p38 levels, whereas no differences in p38 and unphosphorylated and phospho-JNK1/2 expression were observed (Fig. 2C).

### Effect of Gefitinib on Phosphorylation of ERKs and Akt

To define the cellular bases of the differential cell response to gefitinib in the studied cell lines, we examined

**Table 2. Apoptosis induced by gefitinib, U0126, and platinum compounds in ovarian carcinoma cell lines**

| Drug (μmol/L) | Apoptotic cells (%) | | |
|---|---|---|---|
| | IGROV-1 | IGROV-1/OHP | IGROV-1/Pt1 |
| Gefitinib | | | |
| 0 | 5.67 ± 1.7 | 3.04 ± 2.3 | 2.49 ± 2.1 |
| 0.3 | 31.12 ± 7.5 | 19.90 ± 1.8 | 2.62 ± 1.5 |
| 1.0 | 39.30 ± 0.6 | 14.29 ± 0.9 | 2.11 ± 1.2 |
| 3.0 | 47.68 ± 5.0 | 19.93 ± 3.2 | 6.58 ± 2.1 |
| U0126 | | | |
| 0 | 5.63 ± 0.7 | 1.43 ± 1.1 | 3.81 ± 0.5 |
| 0.3 | 15.76 ± 0.5 | 1.93 ± 0.2 | 6.23 ± 1.7 |
| 1.0 | 13.62 ± 0.5 | 2.93 ± 0.5 | 9.62 ± 1.3 |
| OHP | | | |
| 0 | 2.10 ± 1.3 | 1.69 ± 0.1 | 2.01 ± 0.5 |
| 200 | 49.45 ± 2.2 | 1.15 ± 1.0 | 2.94 ± 1.2 |
| cDDP | | | |
| 0 | 4.35 ± 1.2 | 1.30 ± 0.9 | 1.01 ± 0.5 |
| 30 | 59.60 ± 3.2 | 1.92 ± 1.2 | 1.29 ± 1.3 |

NOTE: Drug-induced apoptosis was measured by Annexin V binding assay after 72 h exposure to gefitinib or U0126. For platinum compounds, cells were exposed for 1 h to 200 μmol/L OHP or 30 μmol/L cDDP and harvested 48 h later. Mean ± SD of three independent experiments.

the effect of the drug on phosphorylation of ERK1/2 and Akt, which are downstream events of the EGFR-activated pathway (Fig. 3A). A comparison of the effects of gefitinib at equitoxic concentrations ($IC_{50}$, 0.3 μmol/L for IGROV-1, 3 μmol/L for IGROV-1/OHP, and 10 μmol/L for IGROV-1/Pt1; $IC_{80}$, 10 μmol/L for IGROV-1 and 30 μmol/L for IGROV-1/OHP and IGROV-1/Pt1) revealed a substantial reduction of Akt phosphorylation in both IGROV-1 and IGROV-1/OHP cells. The comparison of the effect at an equimolar concentration (10 μmol/L) indicated that IGROV-1 cells were more sensitive to drug-induced inhibition of Akt phosphorylation. Inhibition of phosphorylation of ERK1/2 was achieved in both cell systems after 24-h exposure, but the effect was more persistent in IGROV-1 cells. Again, a marginal inhibition of Akt phosphorylation was observed in IGROV-1/Pt1 cells, but the effect was less marked than that found in the parental cells already at 24 h. In contrast, no appreciable inhibition of ERK1/2 phosphorylation was observed in the resistant subline.

### Modulation of Sensitivity to Gefitinib by MAPK Inhibitor Pretreatment

To explore the efficacy of the combination of a MAPK inhibitor and gefitinib in cells with reduced sensitivity to gefitinib, we did combination studies in IGROV-1/OHP and IGROV-1/Pt1 cells as well as in parental cells (Fig. 3B). When resistant cells were pretreated for 4 h with subtoxic concentrations of U0126 (0.1 and 0.3 μmol/L) and then gefitinib was added for 72 h, a marked increase in sensitivity to gefitinib was observed in both IGROV-1/OHP and IGROV-1/Pt1 cells. Differently, a marginal modulation was found in IGROV-1 cells under similar conditions (that is, combination with subtoxic concentrations of MAPK inhibitor).

### Effect of cDDP on Stress-Activated Kinases

We examined the effect of cDDP exposure on p38, Akt, ERK1/2, and JNK1/2 pathways. Western blot analysis indicated that exposure to equitoxic concentrations of cDDP (around $IC_{90}$, 60 μmol/L for IGROV-1, 300 μmol/L for IGROV-1/OHP, and 600 μmol/L for IGROV-1/Pt1) induced p38 phosphorylation only in resistant cells (Fig. 3C). In contrast, cDDP produced an appreciable reduction of ERK1/2 phosphorylation in all cell lines. A partial induction of phospho-JNK2 was documented in IGROV-1/OHP and IGROV-1/Pt1 cells (Fig. 3C).

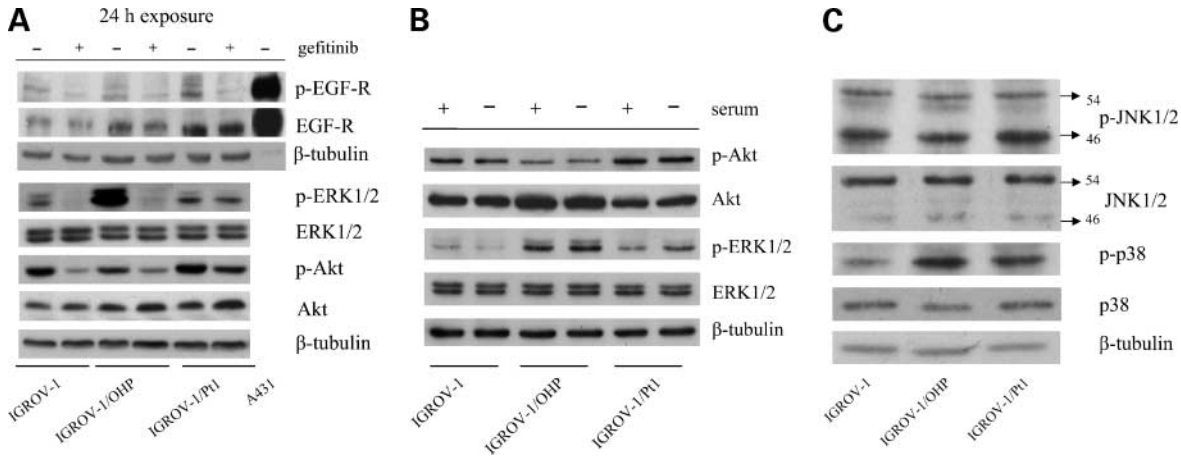


**Figure 2.** **A,** expression of unphosphorylated and phospho-EGFR, phospho-ERK1/2, and phospho-Akt in parental IGROV-1 and platinum-resistant IGROV-1/Pt1 and IGROV-1/OHP cells treated with gefitinib. Cells were exposed to 10 μmol/L gefitinib for 24 h and then harvested for Western blot analysis. Control loading is shown by β-tubulin. Cell lysates from A431 cells were used as positive control. **B,** Western blot analysis of unphosphorylated and phospho-Akt and phospho-ERK1/2 in ovarian carcinoma cell lines. Cells were grown in standard conditions (10% FCS) or serum starved for 24 h and then harvested for Western blot analysis. Control loading is shown by β-tubulin. **C,** Western blot analysis of unphosphorylated and phospho-JNK1/2 and phospho-p38 in ovarian carcinoma cell lines. Control loading is shown by β-tubulin.

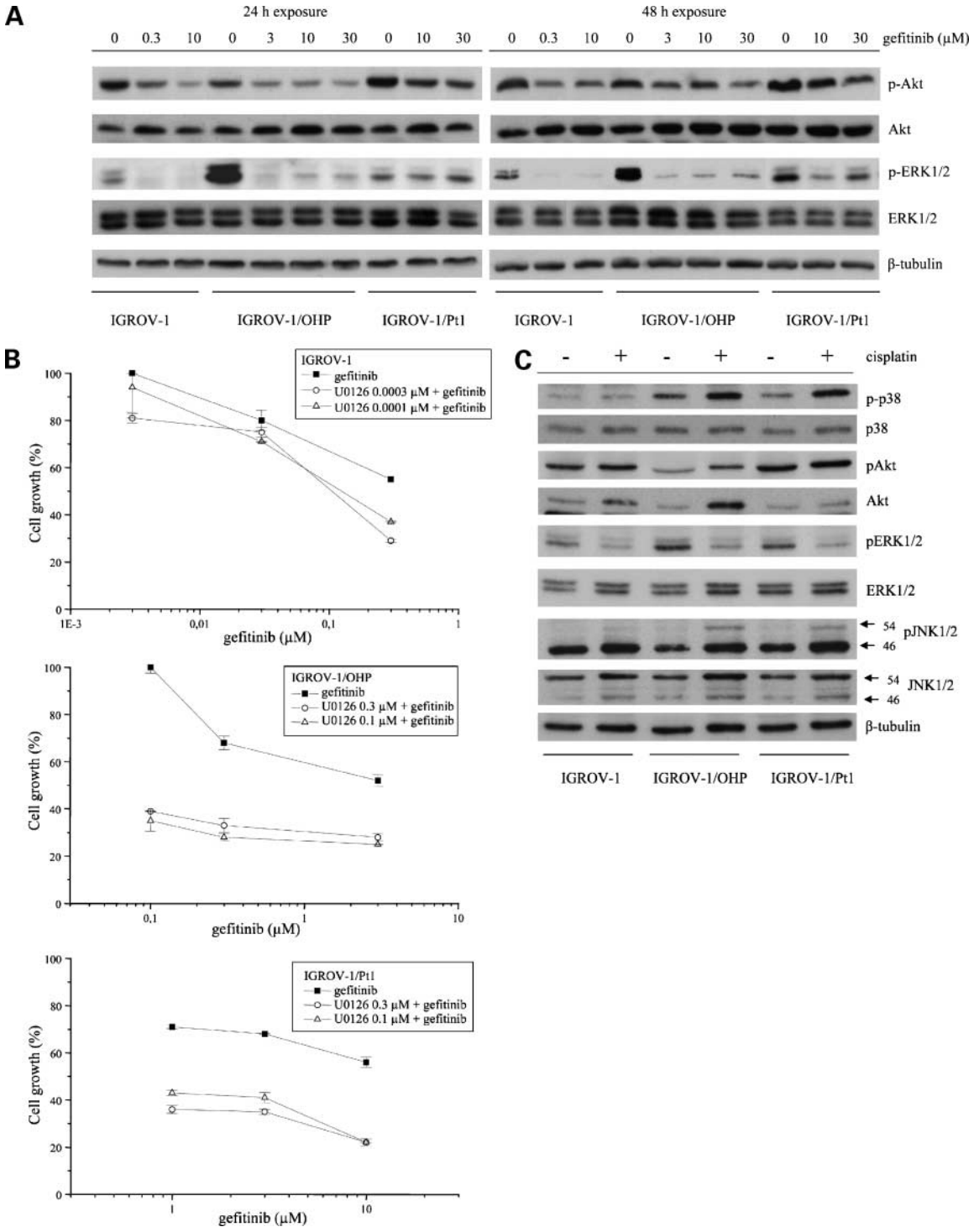


**Figure 3.** **A,** Western blot analysis of unphosphorylated and phospho-EGFR, phospho-Akt, and phospho-ERK1/2 in ovarian carcinoma cell lines treated with gefitinib. Cells were exposed to concentration corresponding to $IC_{50}$ (0.3 μmol/L for IGROV-1, 3 μmol/L for IGROV-1/OHP, and 10 μmol/L for IGROV-1/Pt1) and $IC_{80}$ (10 μmol/L for IGROV-1 and 30 μmol/L for IGROV-1/OHP and IGROV-1/Pt1) for 24 or 48 h and then harvested for Western blot analysis. **B,** modulation of sensitivity to gefitinib by U0126. Cell lines were pretreated for 4 h with U0126 (0.0003 or 0.0001 μmol/L for IGROV-1 and 0.3 or 0.1 μmol/L for IGROV-1/OHP and IGROV-1/Pt1), corresponding to subtoxic concentrations (around $IC_{10}$) and then gefitinib was added for 72 h. Cell sensitivity was assessed by growth inhibition assay. **C,** Western blot analysis of unphosphorylated and phospho-p38, phospho-Akt, phospho-ERK1/2, and phospho-JNK1/2 after cDDP exposure in ovarian carcinoma cell lines. Cells were treated with concentration corresponding to $IC_{90}$ (60 μmol/L for IGROV-1, 300 μmol/L for IGROV-1/OHP, and 600 μmol/L for IGROV-1/Pt1) for 1 h and then harvested for Western blot analysis. Control loading is shown by β-tubulin.

Because cDDP has been shown to induce p38 activation leading to EGFR phosphorylation and internalization and thus switching signaling pathways from proliferation into survival (7), we investigated the effect of cDDP on EGFR localization. Immunofluorescence analysis of EGFR showed that in parental cells the receptor was mainly localized at the plasma membrane. cDDP treatment induced an alteration of cellular localization of the receptor, consistent with induction of internalization (Fig. 4). The effect was more evident in the resistant sublines in which EGFR appeared already delocalized in untreated cells. Western blot analysis of untreated and cDDP-treated cells following cell fractionation into nuclear and cytoplasmic fractions suggested a lack of drug-induced EGFR translocation to the nucleus (data not shown).

## Discussion

In the present study, we employed two ovarian cancer cell lines resistant to cDDP and OHP, in which development of resistance to platinum drugs was associated with increased DNA damage tolerance and resistance to apoptosis. The

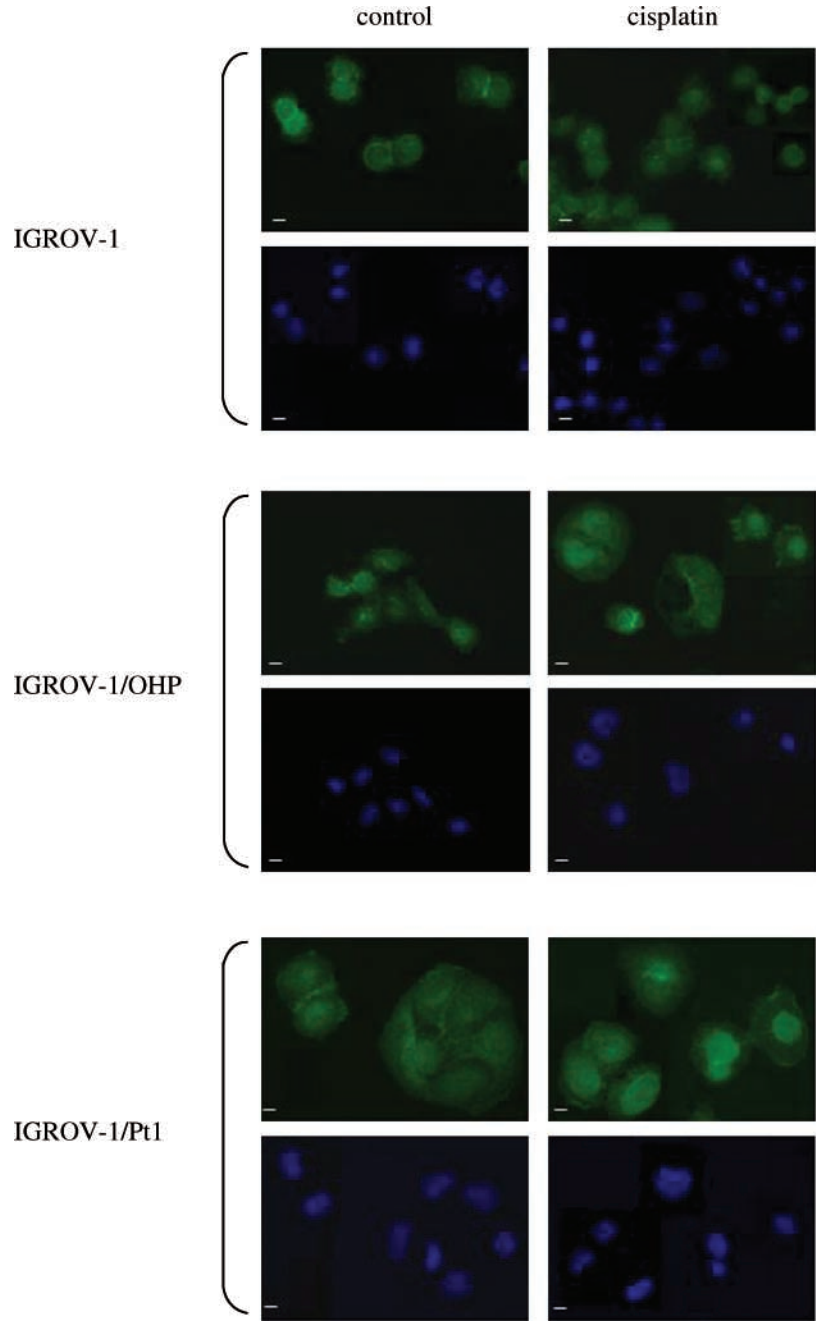


**Figure 4.** EGFR localization in IGROV-1, IGROV-1/OHP, and IGROV-1/Pt1 cell lines. Cells were treated with cDDP concentrations corresponding to $IC_{90}$ (60 μmol/L for IGROV-1, 300 μmol/L for IGROV-1/OHP, and 600 μmol/L for IGROV-1/Pt1) for 1 h and then processed for immunofluorescence assay. Bar, 5 μm.

studied cell systems exhibited markedly reduced sensitivity to the antiproliferative effects of gefitinib irrespective of the platinum compound used for selection (Table 1). The observed behavior was associated with a reduced susceptibility to apoptosis by the EGFR inhibitor (Fig. 1B; Table 2).

Our study shows the absence of EGFR gene mutations that in other reports have been associated with sensitivity/resistance to treatment with gefitinib (21, 24). Such findings and the observation that gefitinib induced down-regulation of EGFR phosphorylation even in resistant cell lines suggest that reduced sensitivity to gefitinib is related to downstream events (Fig. 2A). Indeed, phospho-ERK1/2 levels were increased in the two resistant sublines (Fig. 2B). Such an increase was not due to overexpression of protein phosphatases (that is, MKP-1 and MKP-3; data not shown), different from what observed in other reports (25, 26). The observation of ERK1/2 activation in resistant cells is consistent with decreased sensitivity to the MEK1/2 inhibitor U0126. Also in other model systems, cells with high phospho-ERK1/2 levels have shown reduced sensitivity to such a compound (27). Although the precise link between phospho-ERK1/2 levels and growth inhibition by MAPK inhibitors has not been conclusively defined and may involve both EGFR-dependent and EGFR-independent pathways (28), an efficient inhibition of a highly expressed survival factor is not easily achievable.

Moreover, under our experimental conditions, gefitinib was less effective in inhibiting ERK1/2 phosphorylation in both resistant sublines IGROV-1/OHP and IGROV-1/Pt1. In IGROV-1/OHP cells, the drug produced a marginal down-regulation of phospho-Akt levels compared with what observed in IGROV-1 (Fig. 3A). Such a behavior, consistent with the higher degree of resistance to gefitinib of IGROV-1/Pt1 cells, supports that deregulation of ERK1/2 and Akt is implicated in providing survival advantages. In fact, pretreatment of the two resistant sublines with the MAPK inhibitor U0126 restored sensitivity to gefitinib as shown by growth inhibition assays (Fig. 3B).

A peculiar observation of the present study was the different localization of EGFR in sensitive and resistant cells (Fig. 4). Indeed, a prevalent localization at the plasma membrane was observed in IGROV-1 cells, whereas in the resistant sublines the pattern of EGFR staining suggested that the receptor was mainly internalized. Such a behavior did not reflect nuclear localization in untreated or in drug-treated cells (data not shown). Because cDDP has been reported to induce EGFR internalization mediated by p38 phosphorylation (7), the subcellular localization is consistent with the observed increase of p38 activation in cDDP-resistant cells. Moreover, because internalized EGFR appears still able to activate Akt and internalization is independent of the receptor activation (7), a high constitutive activation of p38 may also account for the marginal down-regulation of phospho-Akt observed in IGROV-1/OHP and IGROV-1/Pt1 cells treated with gefitinib. Indeed, the persistent activation of Akt and ERK1/2 pathways has been related to lack of sensitivity to EGFR inhibitors in non-small cell lung cancer (29). Because ERK1/2 is implicated in signal transduction processes mediated by EGFR, a plausible explanation of the observed cross-resistance between platinum compounds and gefitinib is that activation of ERK1/2 in the resistant variants modulates factors that can provide a survival signal under platinum-induced stress conditions. Such an interpretation is consistent with previous studies showing that inhibition of ERK1/2 activity increases sensitivity to cDDP in ovarian carcinoma SKOV-3 cells (30, 31). However, the role of ERK1/2 in cell response to cDDP appears dependent on the cellular context (e.g., origin of cell lines) and on the experimental conditions (that is, drug concentration and exposure times) as shown in reports supporting a requirement for ERK1/2 activation in cDDP-induced apoptosis (32, 33). Recent studies indicate that pharmacologic inhibition of ERK1/2 attenuates the cytotoxicity of cDDP in some cell types (34).

Our results support that, among multiple alterations acquired during the development of resistance to platinum drugs, deregulation of signaling pathways activated by EGFR, which account for reduced response to gefitinib, may be implicated in the resistant phenotype. In particular, activation of survival signals could contribute to the pattern of cross-resistance, including cross-resistance between platinum compounds and gefitinib. Therefore, the therapeutic potential of approaches directed at specific targets could be limited by expression or activation of protective prosurvival pathways. These observations may have implications in a rational design of novel therapeutic strategies in the treatment of ovarian cancer.



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