

Featured Article

Modulation of DNA Repair *In vitro* after Treatment with Chemotherapeutic Agents by the Epidermal Growth Factor Receptor Inhibitor Gefitinib (ZD1839)

Benjamin Friedmann,¹ Martyn Caplin,²
John A. Hartley,³ and Daniel Hochhauser¹

¹Department of Oncology, Royal Free and University College Medical School, University College London; and ²Department of Gastroenterology, Royal Free Hospital, London, United Kingdom; ³Cancer Research United Kingdom Drug-DNA Interactions Research Group,

ABSTRACT

Purpose: The epidermal growth factor receptor (EGFR) is commonly expressed in human tumors and provides a target for therapy. Gefitinib (Iressa, ZD1839) is a quinazoline derivative that inhibits EGFR tyrosine kinase activity. Gefitinib demonstrated anticancer efficacy *in vivo*, and although experiments *in vitro* have suggested that inhibition of EGFR modulates the activity of chemotherapeutic agents, the mechanism of this interaction is unclear. We investigated mechanisms for this modulation.

Experimental Design: The antiproliferative effect of gefitinib alone or combined with cisplatin, melphalan, and etoposide was determined in a human breast (MCF-7) cancer cell line. Using the alkaline single-cell gel electrophoresis (comet) assay, we investigated kinetics of DNA damage and repair after treatment with the chemotherapeutic drugs combined with gefitinib. To investigate whether the phosphatidylinositol 3'-kinase pathway was contributing to repair-inhibition produced by gefitinib, cells were exposed to chemotherapy in combination with the phosphatidylinositol 3'-kinase inhibitor LY294002.

Results: A superadditive (synergistic) increase in growth inhibition for combined treatment with gefitinib was found for cisplatin and etoposide, but not with melphalan. There was delayed repair of DNA strand breaks after treatment with etoposide combined with gefitinib, and repair of DNA interstrand cross-links produced by cisplatin is delayed in combination with gefitinib. Inhibition of cell prolifer-

ation and DNA repair was identical in cells treated with LY294002. Immunoprecipitation of cell extracts demonstrated that after exposure to gefitinib, there was an association between EGFR and DNA-PK_{CS}.

Conclusion: Gefitinib acts through inhibition of repair of cisplatin and etoposide-induced DNA damage; this effect is mimicked by inhibitors of the phosphatidylinositol 3'-kinase suggesting similar mechanisms of action.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is commonly expressed at high levels in many human cancers including breast, non-small cell lung, and colon (1). EGFR (erbB1) is a member of the family of erbB receptors, the structure of which includes extracellular ligand binding, *trans*-membrane, and intracellular tyrosine kinase domains (2). Activation of the EGFR pathway results in downstream signaling including the lipid kinase phosphatidylinositol 3'-kinase and the serine/threonine kinase pathways (3). This signaling cascade has been implicated in a variety of alterations including proliferation, angiogenesis, and metastasis. EGFR expression increases with tumor progression and is an independent prognostic factor for a variety of malignancies including breast cancer (4).

In view of the importance of the EGFR pathway in human cancer, several approaches to the inhibition of EGFR have been developed with the aim of blocking cancer cell proliferation and promoting apoptosis. These strategies include antibodies blocking the receptor-ligand interaction and small molecules blocking dimerization of the intracellular kinase domain and hence activation of consequent pathways (5, 6).

Gefitinib [Iressa (AstraZeneca, Macclesfield, United Kingdom), ZD1839] is a quinazoline derivative that inhibits EGFR tyrosine kinase activity by competitively inhibiting the ATP-binding domain (7). Gefitinib has demonstrated anticancer efficacy *in vivo* in non-small cell lung cancer (8) and is currently licensed for clinical use. Experiments *in vitro* with several cancer cell types have shown that inhibition of EGFR by antisense mRNA or with gefitinib can increase the cytotoxic effects of several chemotherapeutic agents with differing structure and mode of activity (9, 10); however, the mechanisms underlying these interactions are unclear. Clinical studies have provided additional support for potential interactions between EGFR inhibition and chemotherapy. For example, a recent study with the anti-EGFR antibody cetuximab in irinotecan-refractory colon cancer demonstrated that additional responses to irinotecan could be achieved (11). The mechanism of this resensitization remains unclear, as does the optimal combination of these agents in the treatment of chemotherapy-naïve patients.

To understand the effects of EGFR inhibition on modulat-

Received 3/26/04; revised 6/15/04; accepted 6/22/04.

Grant support: The Times 'Quiet Cancer' Appeal.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Daniel Hochhauser, Department of Oncology, Royal Free and University College School of Medicine, University College London, Gower Street Campus, 91 Riding House Street, London W1W 7BS, United Kingdom. Phone: 44-20-7679-9326; Fax: 44-20-7436-2956; E-mail: d.hochhauser@ucl.ac.uk.

©2004 American Association for Cancer Research.

ing chemotherapeutic drug activity, we investigated the interaction of gefitinib with the widely used drugs cisplatin, etoposide, and melphalan. We investigated the kinetics of DNA damage and repair after treatment with these chemotherapeutic drugs, alone and in combination with gefitinib using the alkaline single-cell gel electrophoresis (comet) assay. There was a significant delay in repair of DNA strand breaks resulting from treatment with etoposide when combined with gefitinib. Additionally, inhibition of repair of cisplatin-induced cross-links was noted with gefitinib. Studies using inhibitors of the phosphatidylinositol 3'-kinase pathway gave similar results in proliferation and repair assays, suggesting involvement of this pathway in the inhibition of repair by these agents.

MATERIALS AND METHODS

Materials. Clinical grade gefitinib was kindly provided by AstraZeneca. Cisplatin, etoposide, and melphalan were purchased from Sigma-Aldrich (Dorset, United Kingdom).

Cell Lines and Culture Conditions. MCF-7 cells (obtained from CR-United Kingdom London Research Institute, London, United Kingdom) were grown in Earle's minimal essential medium (Autogen Bioclear, Wiltshire, United Kingdom) supplemented with 10% fetal calf serum, 1% glutamine, and 0.1% non-essential amino acids and incubated at 37°C in 5% CO₂. For growth inhibition assays, 5 × 10³ cells were seeded into 96-well microtiter plates and left for 48 hours. For single-agent studies, drugs were then added at a range of concentrations to triplicate wells and left in contact for 5 days. For combination studies, drugs were either added concomitantly for 5 days or sequentially by adding each drug in turn for 24 hours, followed by 48 hours in drug-free growth medium. Control wells were treated in the same way with aspiration at each 24-hour period. For combination experiments, gefitinib was added at concentrations producing 10 and 20% inhibition of proliferation, to a range of concentrations for the chemotherapeutic agent. Cytotoxicity was assessed using the sulforhodamine B assay (12). At the end of the incubation period, cell numbers were compared in treated *versus* control wells by fixing in ice-cold 10% (w/v) trichloroacetic acid (20 minutes) and staining with 0.4% sulforhodamine B in 1% (v/v) acetic acid (20 minutes). The mean absorbance at 540 nm for each drug concentration was expressed as a percentage of the control untreated well absorbance.

Isobologram Analysis. To assess whether a combination dose of any given chemotherapeutic agent with gefitinib is synergistic or additive, the isobologram method was used as described previously (13). An IC₅₀ was selected, and doses of each drug alone that give this effect were plotted as axial points in a Cartesian plot.

The straight line connecting their IC₅₀ is the locus of points (dose pairs) that will produce this effect in a simply additive combination. Thus, IC₅₀ for two drugs added together lying on the line have an additive effect. Above this line, they are subadditive (antagonistic), and below the line, superadditive (synergistic).

Single-Cell Gel Electrophoresis (Comet) Assay. MCF-7 cells were treated with etoposide and gefitinib for 24 hours at 37°C. The DNA damage in the form of strand breaks was assessed using the single-cell gel electrophoresis (comet) assay as described previously (14). All procedures were carried out on ice and in subdued lighting. All chemicals used

were obtained from Sigma Chemical (Poole, United Kingdom) unless otherwise stated. In brief, cells were embedded in 1% agarose on a precoated microscope slide, lysed for 1 hour in lysis buffer [100 mmol/L disodium EDTA, 2.5 mol/L NaCl, and 10 mmol/L Tris-HCl (pH 10.5)] containing 1% Triton X-100 (added immediately before analysis), and washed every 15 minutes in distilled water for 1 hour. Slides were then incubated in alkali buffer [50 mmol/L NaOH and 1 mmol/L disodium EDTA (pH 12.5)] for 45 minutes, followed by electrophoresis in the same buffer for 25 minutes at 18 V (0.6 V/cm), 250 mA. The slides were finally rinsed in neutralizing buffer [0.5 mol/L Tris-HCl (pH 7.5)] and then saline. After drying, the slides were stained with propidium iodide (2.5 μg/mL) for 30 minutes and then rinsed in distilled water. Images were visualized with the use of a Nikon inverted microscope with high-pressure mercury light source (Nikon United Kingdom Limited, Kingston Upon Thames, United Kingdom), 510 to 560 nm excitation filter, and 590 nm barrier filter at ×20 magnification. Images were captured by using an on-line charge-couple device camera and analyzed with Komet Analysis software (Kinetic Imaging, Liverpool, United Kingdom). For each duplicate slide, 25 cells were analyzed. DNA damage was measured by the increase in the tail moment, a function of the amount of DNA in the tail and the length of the tail (14). Detection and measurement of DNA interstrand cross-links was achieved using a modification of the comet assay (15). Immediately before analysis, cells were irradiated (12 Gy) to deliver a fixed number of random DNA strand breaks. The tail moment for each image was calculated by using the Komet Analysis software as the product of the percent DNA in the comet tail and the distance between the means of the head and tail distributions, based on the definition of Olive *et al.* (16). Cross-linking was expressed as the percent decrease in tail moment compared with irradiated controls, calculated by the following formula:

Percentage of decrease in tail moment

$$= [1 - (\text{TMdi} - \text{TMcu}/\text{TMci} - \text{TMcu})] \times 100$$

where TMdi equals tail moment of drug-treated irradiated sample, TMcu equals tail moment of untreated unirradiated control, and TMci equals tail moment of untreated irradiated control.

Immunoprecipitation and Western Blotting. Cells were washed twice in PBS and scraped into lysis buffer [radioimmunoprecipitation assay buffer: 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 250 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 100 μg/mL 4-(2-aminoethyl) benzenesulfonyl fluoride, 17 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 5 μmol/L fenvalerate, 5 μmol/L potassium bisperoxo(1,10-phenanthroline)-oxovanadate(V) (BpVphen), and 1 μmol/L okadaic acid]. Lysates were refined by centrifugation at 14,000 × g for 10 minutes at 4°C, and protein concentrations were determined using a protein assay kit (Bio-Rad, Cambridge, MA).

Two-hundred and fifty μg of protein cell lysates were immunoprecipitated using 2 to 4 μg of antibody (anti-EGFR or anti-DNA-PK_{CS}) overnight at 4°C and then bound to protein G Sepharose. Immune complexes were washed three times in lysis buffer before additional analysis. Cell lysates (15–50 μg) and immunoprecipitates were separated by SDS-

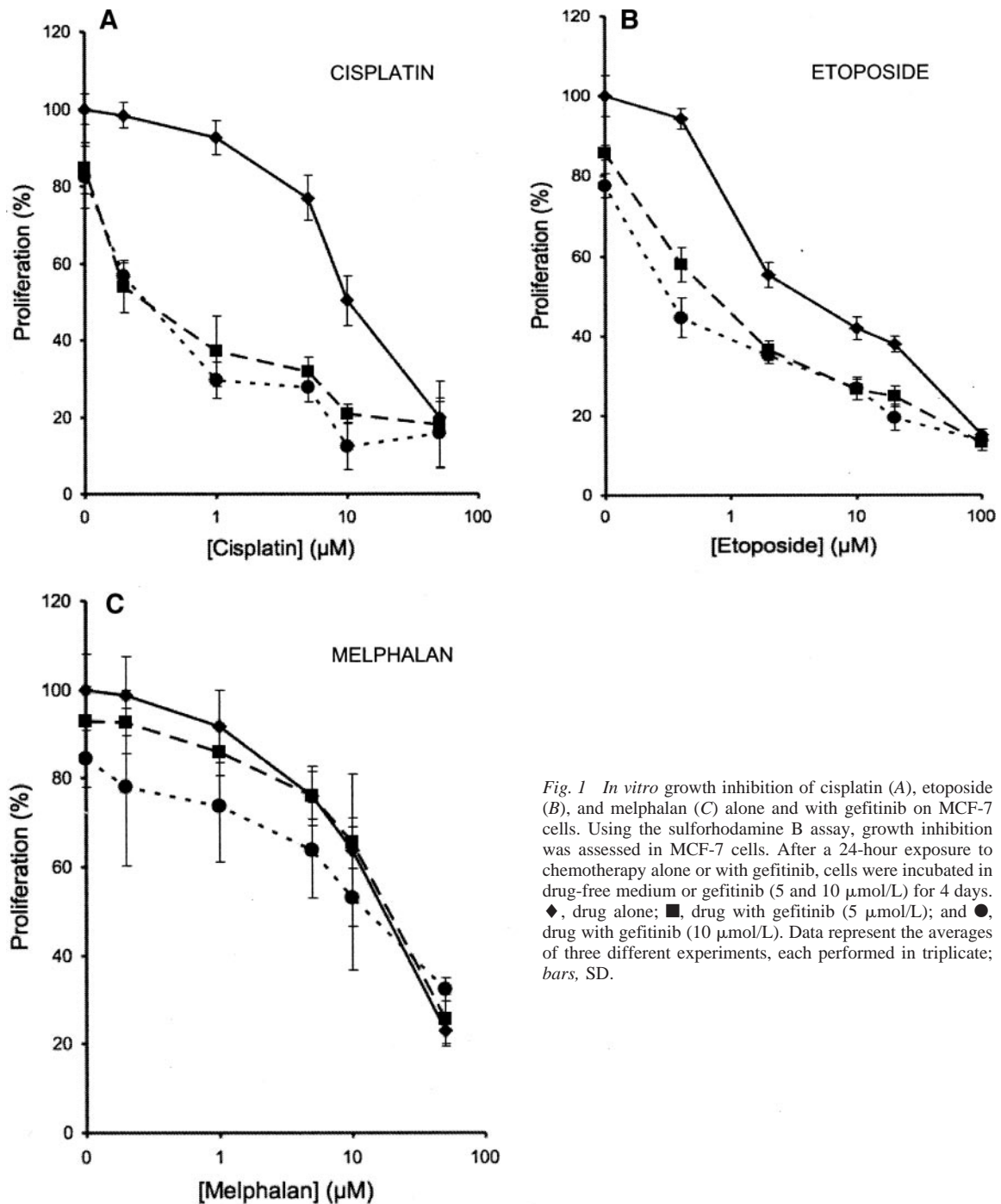


Fig. 1 *In vitro* growth inhibition of cisplatin (A), etoposide (B), and melphalan (C) alone and with gefitinib on MCF-7 cells. Using the sulforhodamine B assay, growth inhibition was assessed in MCF-7 cells. After a 24-hour exposure to chemotherapy alone or with gefitinib, cells were incubated in drug-free medium or gefitinib (5 and 10 μmol/L) for 4 days. ◆, drug alone; ■, drug with gefitinib (5 μmol/L); and ●, drug with gefitinib (10 μmol/L). Data represent the averages of three different experiments, each performed in triplicate; bars, SD.

PAGE, electrophoretically transferred to Immobilon P membrane (Millipore), and probed with appropriate primary antibodies (anti-EGFR or anti-DNA-PK_{CS}) and then horseradish peroxidase-conjugated secondary polyclonal antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Bioclear).

Immunoblotting antibodies were anti-EGFR and horseradish peroxidase-labeled secondary Ab (BD Biosciences), anti-

EGFR (phospho-Tyr⁸⁴⁵; abcam), anti-DNA-PK_{CS}, and anti-tubulin (Sigma).

RESULTS

Gefitinib Treatment Synergizes the Effects of Cisplatin and Etoposide in MCF-7 Cells. There have been several reports on synergistic interactions of several chemotherapeutic

agents and gefitinib (9, 17). We investigated these effects in relation to three commonly used chemotherapeutic agents; cisplatin, etoposide, and melphalan. Treatment of MCF-7 cells with gefitinib alone demonstrated a 50% growth inhibition (IC_{50}) of $17 \pm 0.021 \mu\text{mol/L}$ as measured by a sulforhodamine B proliferation assay (results not shown). This is similar to results obtained in other studies using this cell line (18). The IC_{50} concentration for cisplatin alone was $10 \pm 0.9 \mu\text{mol/L}$. Cells were treated with cisplatin for 24 hours in combination with gefitinib followed by gefitinib alone. There was a synergistic effect on inhibition of cell proliferation when MCF-7 cells were treated with cisplatin in combination with gefitinib. In contrast to cisplatin alone, incubation of cisplatin and $5 \mu\text{mol/L}$ gefitinib resulted in an IC_{50} of $0.2 \pm 0.003 \mu\text{mol/L}$ (Fig. 1A).

Similar experiments were performed with MCF-7 cells using etoposide, a topoisomerase II poison. Cells exposed to etoposide alone demonstrated an IC_{50} of $3.5 \pm 0.08 \mu\text{mol/L}$, whereas in the presence of $5 \mu\text{mol/L}$ gefitinib, the IC_{50} was $0.7 \pm 0.005 \mu\text{mol/L}$ (Fig. 1B). In contrast to the results obtained with cisplatin and etoposide, there was no demonstrable synergy with melphalan in combination with gefitinib with an IC_{50} of $19 \pm 1.08 \mu\text{mol/L}$ (Fig. 1C). Therefore the effects of gefitinib on cell proliferation in combination with chemotherapy are variable with different chemotherapeutic agents. Analysis of these results by an isobologram methodology (13) confirmed that the combinations of gefitinib with cisplatin and etoposide were synergistic in contrast to the result in combination with melphalan (Fig. 2).

Gefitinib Inhibits Repair of Cisplatin and Etoposide-Induced DNA Damage. In view of the effects of gefitinib on the cytotoxicity of cisplatin and etoposide, but not melphalan, we investigated whether this could be modulated at the level of drug-induced DNA damage. To determine effects on DNA damage and repair, we used the single-cell gel electrophoresis (comet) assay (16). This can be used to quantitate DNA strand breaks and repair after exposure to drug. A modification to measure DNA interstrand cross-links has been developed (14, 15). This involves irradiating the cells immediately before analysis to deliver a fixed level of random DNA strand breaks. In the presence of strand breaks, interstrand cross-links retard migration, thus reducing the tail moment compared with the non-cross-linked irradiated control. To examine the kinetics of DNA repair, a brief exposure to etoposide, cisplatin, and melphalan was followed by prolonged incubation with gefitinib.

Quantitation of DNA strand breaks after etoposide treatment has been well characterized. Initial experiments with etoposide confirmed that after exposure of MCF-7 cells to subtoxic concentrations of drug, maximal strand breakage was detectable after 2 hours of exposure (data not shown). There was no alteration in the quantity of strand breaks in cells produced with etoposide alone and in combination with gefitinib (Fig. 3). After removal of drug, strand-break repair was detectable within 30 minutes with almost complete repair after 4 hours. As shown in Fig. 3, exposure of cells to gefitinib alone produced no DNA strand breaks. After combination treatment with both etoposide and gefitinib, there was a marked delay in the rate of repair of DNA strand breaks with 45% of strand breaks remaining at 1 hour. There was persistent DNA damage detectable after 24 hours, in contrast with the result after exposure to etoposide alone. Both cisplatin and melphalan produce

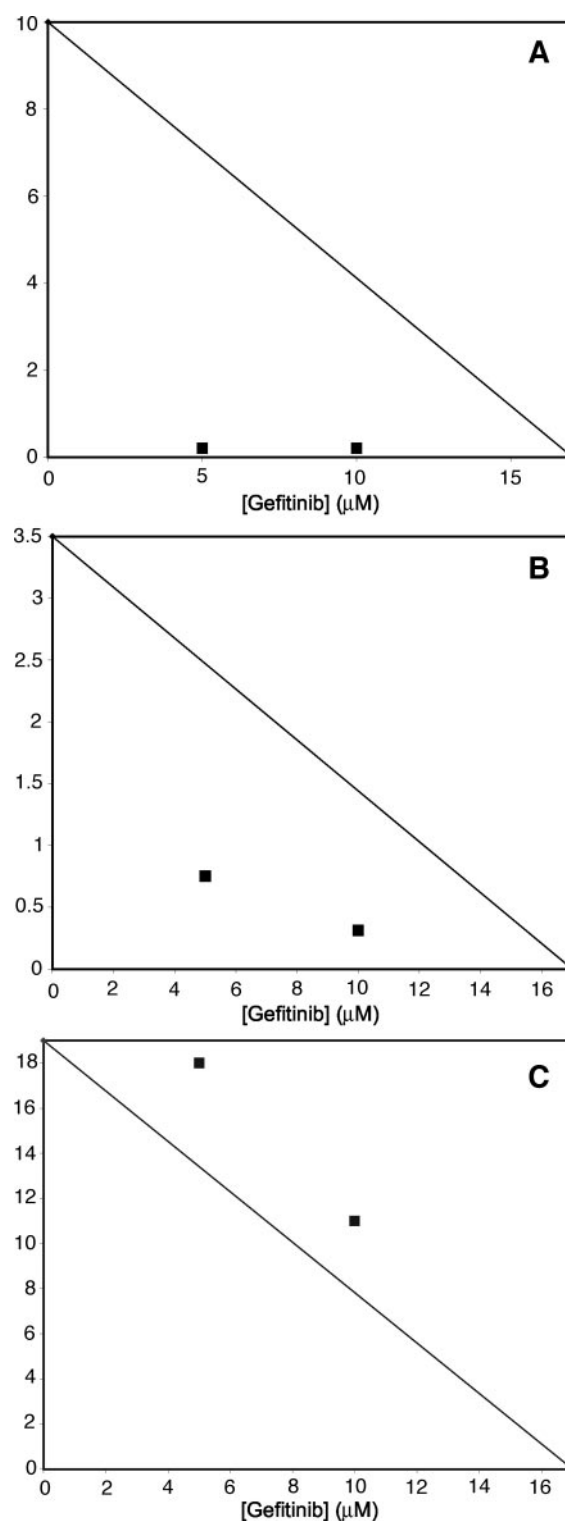


Fig. 2 Isobologram analyses of growth inhibition of cisplatin (A), etoposide (B), and melphalan (C) alone and with gefitinib (5 and 10 $\mu\text{mol/L}$) on MCF-7 cells. The straight line connecting their IC_{50} points (additivity line) is the locus of all dose pairs that, based on these potencies, should give the same effect. A dose pair attaining this effect with lower quantities is superadditive (synergistic), whereas a point appearing close to the line is additive. Dose pairs IC_{50} of drug and gefitinib together.

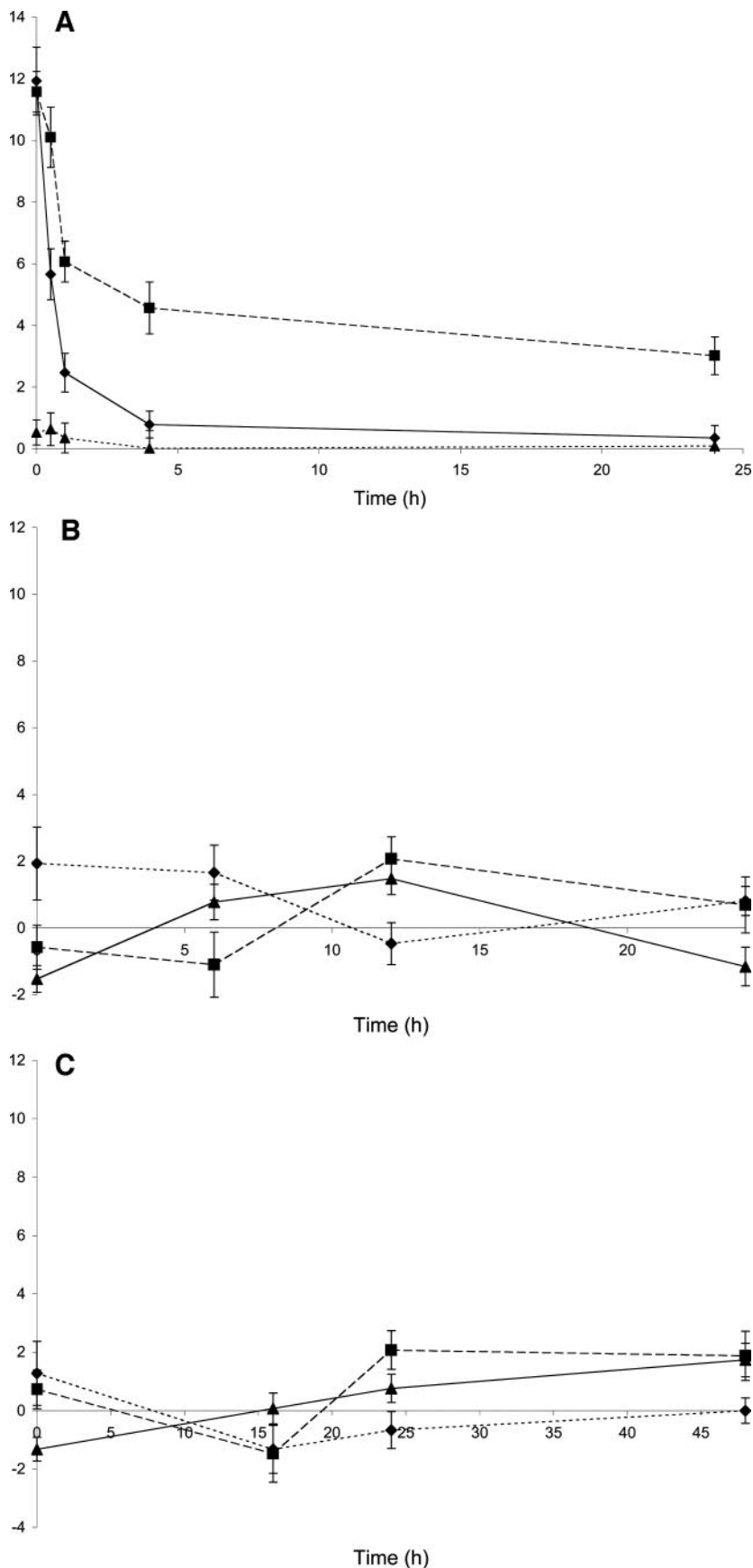
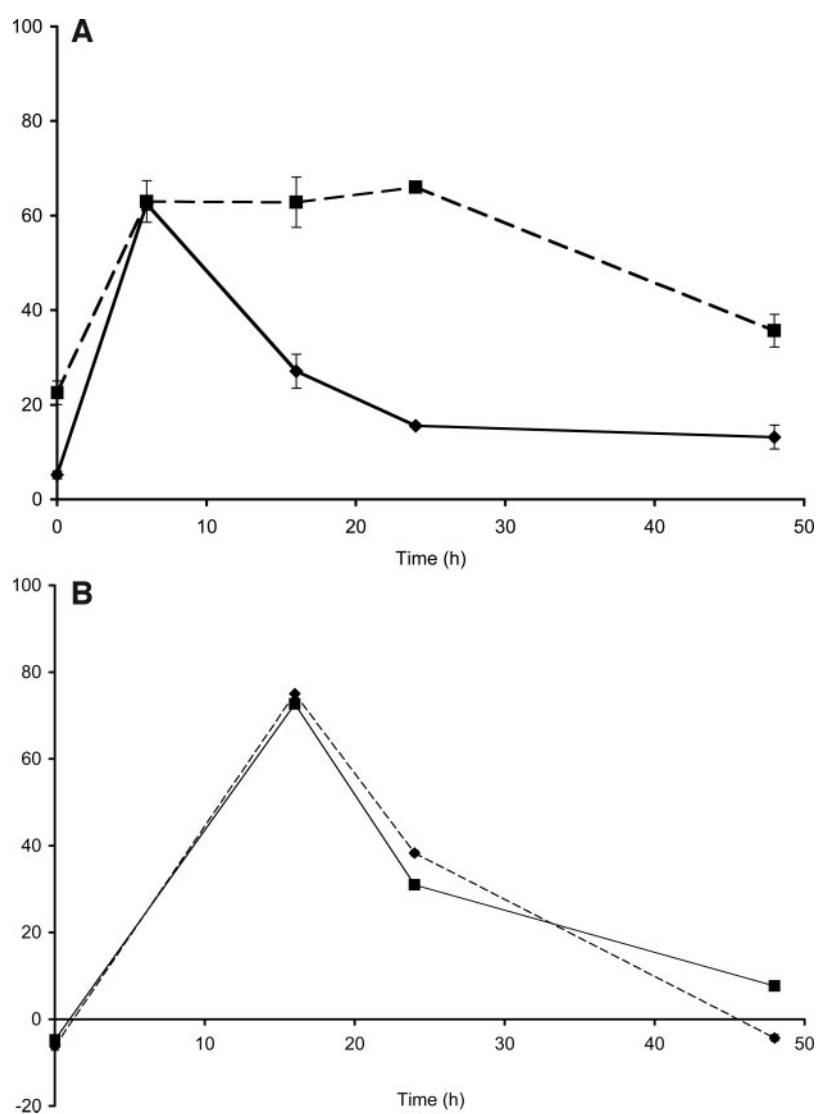


Fig. 3 A, measurement of etoposide-induced DNA strand breaks and their repair alone or in the presence of gefitinib in MCF-7 cells. Cells were treated with etoposide (100 $\mu\text{mol/L}$) and gefitinib (10 $\mu\text{mol/L}$) for 2 hours and then incubated with gefitinib (10 $\mu\text{mol/L}$) alone. Strand-break formation measured as tail moment length (micrometers). ◆, etoposide; ■, etoposide with gefitinib (10 $\mu\text{mol/L}$); and ▲, gefitinib (10 $\mu\text{mol/L}$). B, measurement of cisplatin-induced DNA strand breaks and their repair alone or in the presence of gefitinib in MCF-7 cells. Cells were treated with cisplatin (200 $\mu\text{mol/L}$) and gefitinib (10 $\mu\text{mol/L}$) for 1 hour and then incubated with gefitinib (10 $\mu\text{mol/L}$) alone. Strand-break formation measured as tail moment length (micrometers). ◆, cisplatin; ■, cisplatin with gefitinib (10 $\mu\text{mol/L}$); ▲, gefitinib (10 $\mu\text{mol/L}$). C, measurement of melphalan-induced DNA strand breaks and their repair alone or in the presence of gefitinib in MCF-7 cells. Cells were treated with melphalan (200 $\mu\text{mol/L}$) and gefitinib (10 $\mu\text{mol/L}$) for 1 hour and then incubated with gefitinib (10 $\mu\text{mol/L}$) alone. Strand-break formation measured as tail moment length (micrometers). ◆, melphalan; ■, melphalan with gefitinib (10 $\mu\text{mol/L}$); and ▲, gefitinib (10 $\mu\text{mol/L}$). Data represent the averages of three different experiments; bars, SE.

Fig. 4 A, measurement of cisplatin-induced DNA interstrand cross-links and repair alone and in the presence of gefitinib in MCF-7 cells. Cells were treated with cisplatin (200 $\mu\text{mol/L}$) and gefitinib (10 $\mu\text{mol/L}$) for 1 hour and then incubated with gefitinib (10 $\mu\text{mol/L}$) alone. Interstrand cross-link formation represented as percent decrease in tail moment. \blacklozenge , cisplatin; and \blacksquare , cisplatin with gefitinib (10 $\mu\text{mol/L}$). B, measurement of melphalan-induced DNA interstrand cross-links and repair alone or in the presence of gefitinib in MCF-7 cells. Cells were treated with melphalan (100 $\mu\text{mol/L}$) and gefitinib (10 $\mu\text{mol/L}$) for 1 hour and then replaced with gefitinib (10 $\mu\text{mol/L}$) alone. Interstrand cross-link formation represented as percent decrease in tail moment. \blacklozenge , melphalan; and \blacksquare , melphalan with gefitinib (10 $\mu\text{mol/L}$). Data represent the averages of three different experiments; bars, SE.



no detectable strand breaks alone or in the presence of gefitinib (Fig. 3B and C).

Additional experiments were performed using MCF-7 cells after incubation with cisplatin. Interstrand cross-links have been demonstrated to be critical in the cellular effects of cisplatin (19). The formation and repair of interstrand cross-links formed after a 1-hour exposure to cisplatin were measured. In MCF-7 cells, interstrand cross-links were repaired efficiently with almost complete removal by 24 hours (Fig. 4A). To investigate whether the addition of gefitinib modulated the repair of cisplatin-induced DNA lesions, cells were exposed for 1 hour to cisplatin, after which the amount of DNA damage and the rate of repair were assessed (Fig. 4A). For MCF-7 cells incubated with gefitinib and cisplatin, there was no effect on the quantity of interstrand cross-links produced as compared with cisplatin alone. However, there was clear inhibition of cisplatin-induced interstrand cross-link repair after coincubation with gefitinib as compared with cisplatin alone. In contrast, cells treated with the

combination of gefitinib and cisplatin showed persistent interstrand cross-links at 48 hours after removal of drug. Gefitinib alone produced no interstrand cross-links (data not shown).

The response of MCF-7 cells to melphalan was also assessed. Previous experiments using the sulforhodamine B assay did not demonstrate a synergistic interaction between gefitinib and melphalan on the inhibition of cell proliferation. Comet assays demonstrated that melphalan-induced DNA cross-links peak 16 hours after a 1-hour exposure to drug and are repaired after 48 hours following treatment. In contrast to the results obtained using gefitinib in combination with etoposide and cisplatin, exposure of MCF-7 cells with gefitinib and melphalan resulted in no alteration in the kinetics of DNA repair (Fig. 4B).

To address whether inhibition of DNA damage repair by gefitinib leads to the induction of apoptosis, the annexin V assay was performed. This showed no evidence of apoptosis during the time course of the experiment (data not shown). Therefore the strand breaks are not due to apoptosis.

Inhibition of Phosphatidylinositol 3'-Kinase Pathway and Chemosensitivity in MCF-7 Cells. The phosphatidylinositol 3'-kinase pathway has been implicated in the repair of cisplatin and etoposide-induced DNA lesions (20, 21). To investigate whether the inhibition of repair found with gefitinib could be mediated through this pathway, we exposed MCF-7 cells to the phosphatidylinositol 3'-kinase inhibitor LY294002. Synergy was demonstrated in an sulforhodamine B assay with LY294002 when MCF-7 cells were treated with cisplatin (Fig. 5A). The IC_{50} for cells treated with cisplatin and LY294002 was $0.25 \pm 0.04 \mu\text{mol/L}$, comparable with the combination of cisplatin and gefitinib ($0.2 \pm 0.003 \mu\text{mol/L}$). Experiments in which cells were exposed to the combination of gefitinib, LY294002, and cisplatin did not result in additional inhibition of cellular proliferation with an IC_{50} of $0.20 \pm 0.05 \mu\text{mol/L}$. Interestingly, as with gefitinib, there was no demonstrable synergy for the combination of LY294002 and melphalan (Fig. 5B).

To determine the significance of inhibition of the phosphatidylinositol 3'-kinase pathway in the cellular response to cisplatin, we performed comet assays on cells after exposure to cisplatin and LY294002. A delay in repair of the cisplatin-induced interstrand cross-links was found with kinetics comparable with that found on exposure to gefitinib and cisplatin. The addition of gefitinib to the combination of cisplatin and LY294002 did not further alter the extent of DNA interstrand cross-link formation or the time course for DNA repair (Fig. 6A). These results suggest that the inhibition of repair of cisplatin-induced DNA lesions by gefitinib and LY294002 may occur through the same pathway. Experiments with cisplatin in combination with the phosphatidylinositol 3'-kinase inhibitor wortmannin showed similar effects to LY294002 on inhibition of cellular proliferation and DNA repair (data not shown).

As previously found with gefitinib, exposure of cells to melphalan and LY294002 resulted in no alteration of the DNA repair profile as compared with melphalan alone as assessed by comet assays (Fig. 6B). Therefore the inhibitory effect of LY294002 on DNA repair does not occur with all DNA-interactive chemotherapeutic agents.

Modulation of Epidermal Growth Factor Receptor and DNA-PKcs by Gefitinib. Incubation of cells with cisplatin resulted in increased phosphorylation of EGFR as described previously with other cell lines (Fig. 7A; ref. 22). Treatment of cells with gefitinib resulted in a decrease in phosphorylated EGFR. The increase in phosphorylated EGFR after cisplatin treatment was blocked by incubation with gefitinib. Similar results were obtained using the phospho-tyrosine antibody, PY20 (data not shown).

In view of the results above suggesting involvement of the phosphatidylinositol 3'-kinase pathway in response of cells to gefitinib and cisplatin, we performed immunoblotting of MCF-7 cells after exposure to cisplatin and melphalan and assessed protein levels of the catalytic subunit of DNA-PK (DNA-PK_{CS}). After exposure to gefitinib, there was a reduction in DNA-PK_{CS} levels, although neither cisplatin nor melphalan treatment alone had any effect on DNA-PK_{CS} levels. The reduction in DNA-PK_{CS} levels persisted when cells were exposed to combinations of cisplatin and melphalan with gefitinib (Fig. 7B).

A previous report suggested association of EGFR and DNA-PK_{CS} in cells treated with the anti-EGFR antibody C225

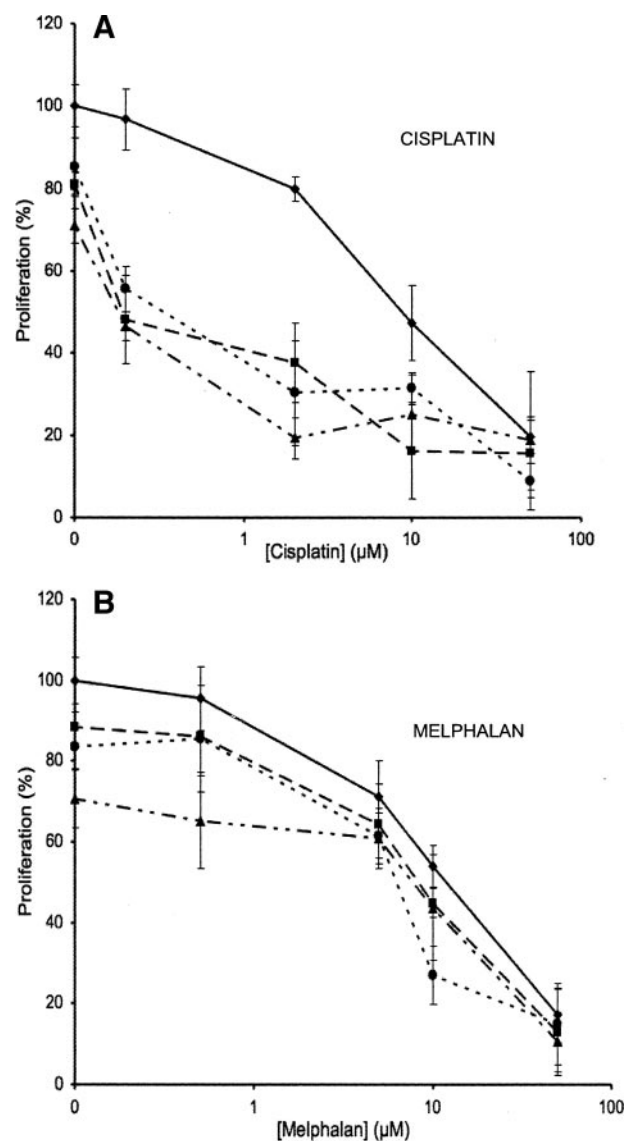
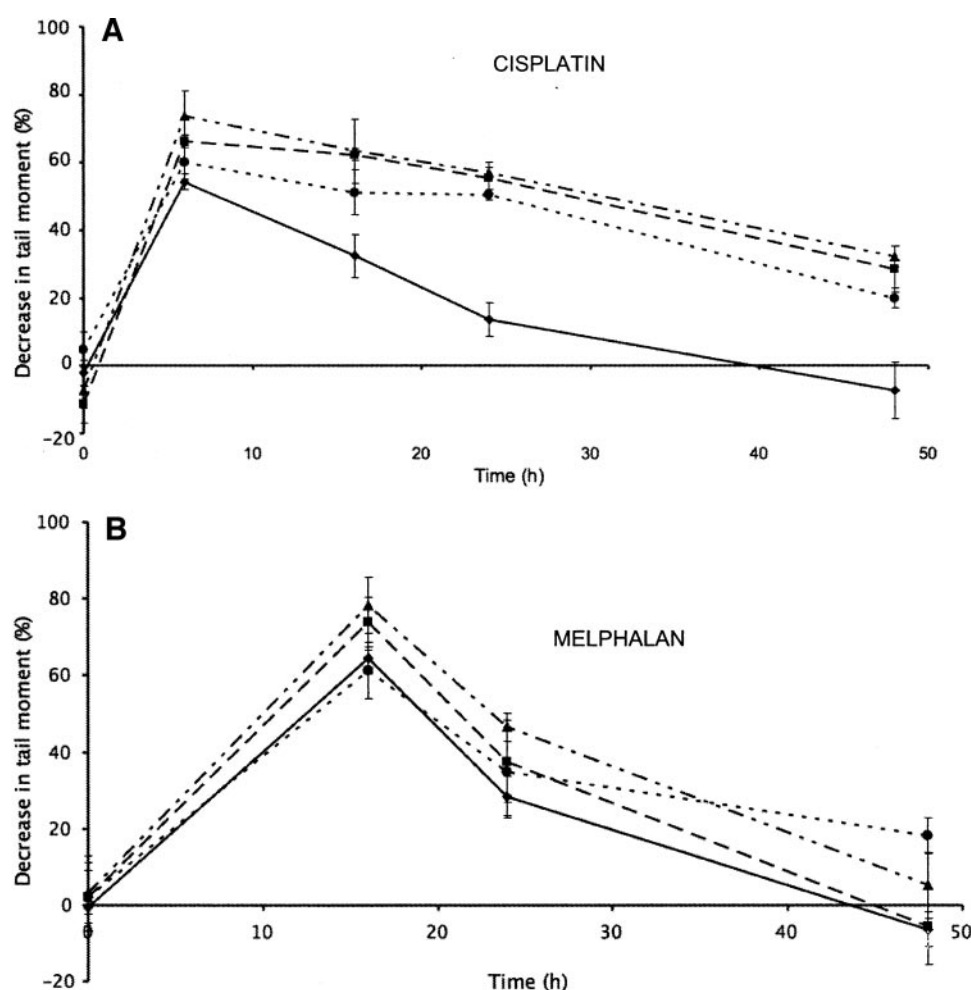


Fig. 5 *In vitro* growth inhibition of cisplatin (A) and melphalan (B) alone and with gefitinib and/or LY294002 of MCF-7 cells. Using the SRB assay, growth inhibition was assessed in MCF-7 cells. After a 24-hour exposure to chemotherapy alone or with gefitinib and/or LY294002, cells were incubated in drug-free medium or gefitinib ($10 \mu\text{mol/L}$) and/or LY294002 ($10 \mu\text{mol/L}$) for 4 days. \blacklozenge , drug alone; \blacksquare , drug with gefitinib ($10 \mu\text{mol/L}$); \bullet , drug with LY294002 ($10 \mu\text{mol/L}$); and \blacktriangle , drug with gefitinib ($10 \mu\text{mol/L}$) and LY294002 ($10 \mu\text{mol/L}$). Data represent the averages of three different experiments, each performed in triplicate; bars, SD.

(23). To investigate whether gefitinib had similar effects on the association of EGFR and DNA-PK_{CS}, we performed immunoprecipitations with EGFR and probed with DNA-PK_{CS} on cell lysates after treatment with gefitinib. As shown in Fig. 7C, an association of EGFR and DNA-PK_{CS} was detectable in untreated cells. However, after treatment with gefitinib, an increased amount of DNA-PK_{CS} was found to be associated with EGFR. A similar result was found when cell extracts were immunoprecipitated with DNA-PK_{CS} and probed with antibody

Fig. 6 Measurement of cisplatin (A) and melphalan (B)-induced DNA interstrand cross-links and repair alone or in the presence of gefitinib and/or LY294002 in MCF-7 cells. Cells were treated with cisplatin (200 $\mu\text{mol/L}$) or melphalan (100 $\mu\text{mol/L}$) and gefitinib and/or LY294002 for 1 hour and then incubated with gefitinib and/or LY294002 alone. At various time points after initial exposure, cells were harvested and analyzed using the comet assay. Interstrand cross-link formation is represented as percent decrease in tail moment. \blacklozenge , drug alone; \blacksquare , drug with LY294002 (10 $\mu\text{mol/L}$); \bullet , drug with gefitinib (10 $\mu\text{mol/L}$); \blacktriangle , drug with gefitinib (10 $\mu\text{mol/L}$) and LY294002 (10 $\mu\text{mol/L}$). Data represent the averages; bars, SE.



to EGFR (Fig. 7D). These results suggest that effects of gefitinib on DNA-PK_{CS} may be mediated through induced association with EGFR.

DISCUSSION

This study demonstrates that gefitinib, a small molecule inhibitor of EGFR, may synergize the effects of DNA-interactive chemotherapeutic drugs by modulation of DNA repair. EGFR is commonly expressed in human cancers through a variety of mechanisms including gene amplification and mutation (1). Activation of the EGFR receptor results in modulation of downstream pathways including the *ras/raf/mitogen-activated protein kinase* cascades. The expression of EGFR in tumors may result in increased rate of cellular proliferation, metastasis, and angiogenesis.

Several strategies for the inhibition of EGFR have undergone clinical evaluation and demonstrated *in vitro* and clinical activity in malignancies including colon and non-small cell lung cancers (8, 11). Experimental studies on cell lines and xenografts have shown synergies between gefitinib and a variety of anticancer agents including chemotherapeutic agents and radiation (24). The basis for these synergies has not been clearly

defined. Inhibition of EGFR has been shown to directly affect the threshold of apoptosis within the cell by mechanisms including caspase activation (25). However, it is unclear as to whether the synergies demonstrated thus far in relation to anticancer activity are specific to particular agents or apply equally to all classes of anticancer drugs.

This study investigated the effects of EGFR inhibition on the modulation of DNA damage in the breast cancer cell line MCF-7. Studies on cellular proliferation confirmed synergies previously noted in other cell lines with cisplatin and etoposide, but this effect was not found with the nitrogen mustard melphalan. The MCF-7 cell line has been noted to be relatively resistant to inhibition of proliferation by gefitinib compared with other cell lines (18). The nature of the DNA damage induced by these agents is well understood. The comet assay is a validated methodology for quantitating DNA damage and repair after exposure to these drugs (14, 15). As discussed above, the formation of strand breaks cannot be accounted for by induction of apoptosis. Initial experiments demonstrated striking inhibition of DNA repair by gefitinib of both DNA strand breaks and interstrand cross-links after exposure to etoposide and cisplatin, respectively. Interestingly, as with the results found in proliferation

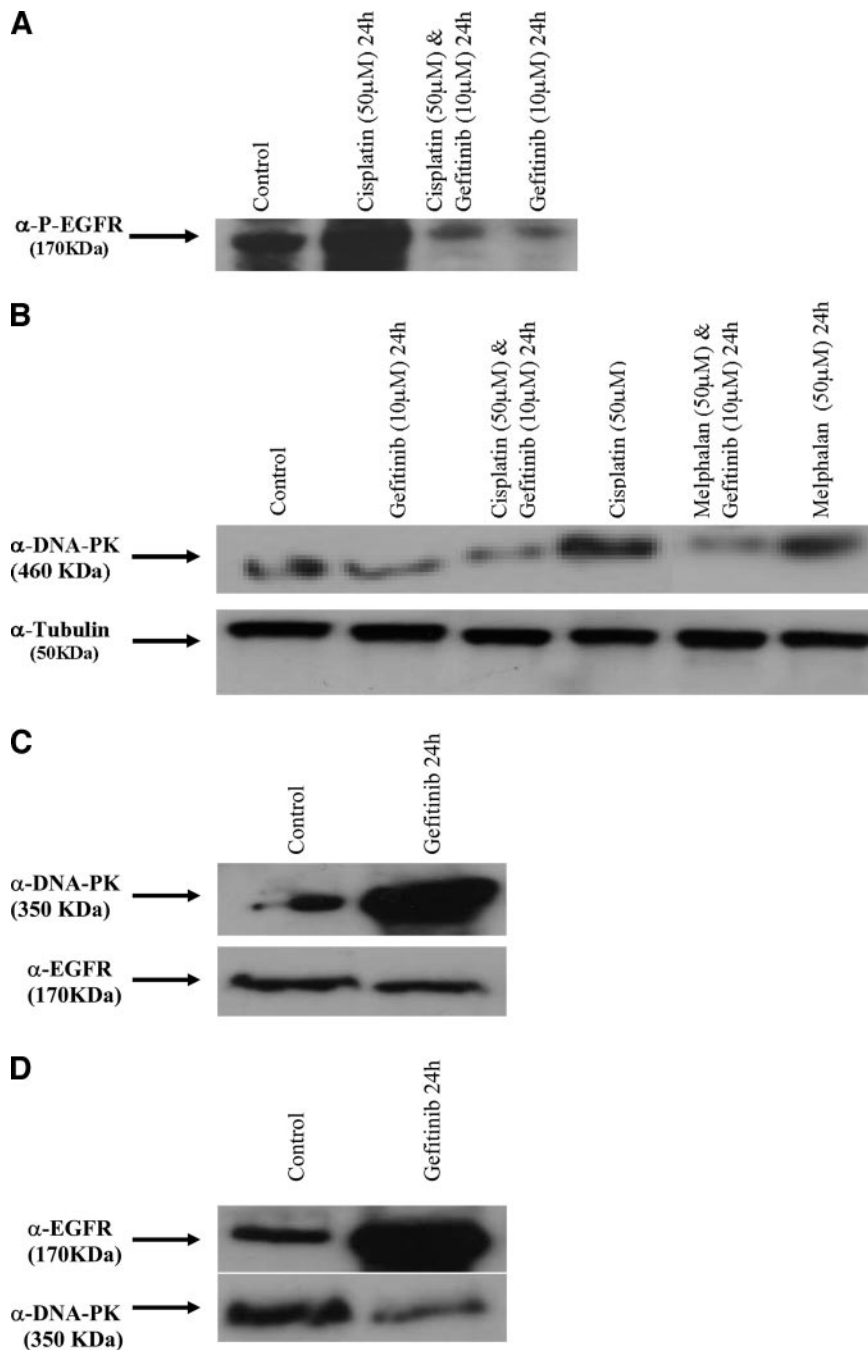


Fig. 7 A, tyrosine phosphorylation of EGFR in MCF-7 cells determined by immunoprecipitation with anti-EGFR and immunoblotted with anti-EGFR (phospho-Tyr⁸⁴⁵) antibody. Cells were treated with cisplatin and gefitinib as described in the figure. B, immunoblotting with anti-DNA-PK_{CS} antibody on MCF-7 cell lysates. Cells were treated with cisplatin, melphalan, and gefitinib as described in the figure. C, Gefitinib induces association of EGFR with DNA-PK. *Top panel*. MCF-7 cells were treated with gefitinib (10 μ mol/L), and lysates were immunoprecipitated with anti-EGFR and immunoblotted with anti-DNA-PK_{CS} antibody. *Bottom panel*. The blot was then stripped and reprobbed with antibody to EGFR. D, treatment as in C. *Top panel*. Lysates were immunoprecipitated with anti-DNA-PK_{CS} and immunoblotted with anti-EGFR. *Bottom panel*. The blot was then stripped and reprobbed with antibody to DNA-PK_{CS}.

assays, there was no modulation of the repair of DNA damage after treatment with melphalan.

To investigate these findings further, we focused on the DNA-PK pathway. DNA-PK is a critical component of the nonhomologous end rejoining nonhomologous end-joining recombination pathway, which has been shown to be responsible for repair of DNA double-strand breaks (26, 27). The DNA-PK complex consists of a heterodimer of the Ku70 and Ku80 proteins and DNA-PK_{CS}. The significance of this pathway in repair of drug-induced DNA damage has been shown for cells

lacking nonhomologous end-joining recombination activity, which are hypersensitive to etoposide (28). The role of nonhomologous end-joining recombination in repair of DNA crosslinks produced by cisplatin is unclear, but cellular sensitization to cisplatin has been found with other inhibitors of DNA-PK (29). Synergy of the effects of cisplatin on cell proliferation was found when cells were exposed to wortmannin and LY294002, which block the phosphatidylinositol 3'-kinase pathway. This was associated with inhibition of repair to a similar extent as that found with gefitinib; as with gefitinib, neither synergistic

effects on cytotoxicity nor effects on DNA repair were found with melphalan. An inhibitory effect of wortmannin on etoposide-induced DNA damage has been demonstrated (20).

There has been contrasting evidence regarding the role of nonhomologous end-joining recombination in repair of melphalan-induced lesions; although this has been demonstrated in hemopoietic cancer cells, there is evidence that it is the homologous recombination pathway that is critical in epithelial tumor cell lines (30, 31). The type and frequency of DNA lesions due to cisplatin differ from those produced by melphalan. In contrast to the repair of interstrand cross-links produced by melphalan, repair of cisplatin interstrand cross-links does not involve the formation of DNA double-strand breaks (32, 33). The mechanisms for the differences in inhibition of repair of melphalan and cisplatin-induced interstrand cross-links warrant additional investigation. There was no difference in the DNA-PK_{CS} level after drug treatment, and therefore this cannot account for the lack of synergy found with gefitinib and melphalan.

The mechanism by which gefitinib might inhibit the non-homologous end-joining recombination pathway is unclear. A study with the antibody C225 directed against EGFR demonstrated a physical interaction between the EGFR and DNA-PK_{CS} (23). The functional significance of this interaction is unclear. A study using a combination of radiation and C225 demonstrated an alteration in the subcellular distribution of DNA-PK with an increase in cytosolic levels and a decrease in nuclear DNA-PK levels (34), whereas other studies have shown a reduction in DNA-PK levels after prolonged exposure to gefitinib (35). EGFR has been shown to directly interact with a variety of cellular proteins, and this may be an important factor in the activity of EGFR-inhibitory strategies.

There is an important need to define the optimal schedule of administration of anticancer drugs with EGFR inhibitors as well as identifying the agents with which these synergies are seen. A study of gefitinib in colon cancer cell lines found a synergistic effect on chemosensitivity and removal of DNA adducts when oxaliplatin was followed by gefitinib; scheduling of oxaliplatin followed by gefitinib resulted in an additive effect at best (36). Interestingly, the synergistic effects of gefitinib and the fluoropyrimidine derivative capecitabine were observed when gefitinib administration preceded drug exposure. Pretreatment with gefitinib reduced cell proliferation and consequently resulted in a 40-fold reduction in the enzyme thymidylate synthase, a key target of fluoropyrimidines (37). Recent clinical evidence suggests that irinotecan-resistant colon cancer can be successfully treated in a cohort of patients with a combination of the EGFR-inhibitory antibody C225 and irinotecan (11).

In conclusion, gefitinib modulates the activity of cisplatin and etoposide by a direct effect on the repair of chemotherapy-induced DNA lesions. These results provide a rationale for additional characterization of the mechanisms of these interactions to inform additional therapeutic trials of EGFR inhibitors. It will be critical to investigate these interactions in a variety of cancer cell lines. Understanding the mechanism of the interaction between EGFR inhibition and chemotherapy will be important in devising novel schedules and combinations for testing in the clinic.

ACKNOWLEDGMENTS

We thank Rob Stein for helpful discussions.

REFERENCES

- Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19:183–232.
- Yarden Y, Sliwkowski MX. Untangling the erbB signaling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000;103:211–25.
- Fox SB, Smith K, Hollyer J, Greenall M, Hastrich D, Harris AL. The epidermal growth factor receptor as a prognostic marker: results of 370 patients and review of 3009 patients. *Breast Cancer Res Treat* 1994;29:41–9.
- Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* 2001;7:2958–70.
- Slichenmyer WJ, Fry DW. Anticancer therapy targeting the erbB family of receptor tyrosine kinases. *Semin Oncol* 2001;28(Suppl 16):67–79.
- Morin MJ. From oncogene to drug: development of small molecule tyrosine kinase inhibitors as anti-tumor and anti-angiogenic agents. *Oncogene* 2000;19:6574–83.
- Sridhar SS, Seymour L, Shepherd FA. Inhibitors of epidermal-growth-factor receptors: a review of clinical research with a focus on non-small-cell lung cancer. *Lancet Oncol* 2003;4:397–406.
- Ciardiello F, Caputo R, Bianco R, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000;6:2053–63.
- Ciardiello F, Caputo R, Troiani T, et al. Antisense oligonucleotides targeting the epidermal growth factor receptor inhibit proliferation, induce apoptosis, and cooperate with cytotoxic drugs in human cancer cell lines. *Int J Cancer* 2001;93:172–8.
- Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau, I, Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med*. 2004 351(4):337–45.
- Skehan P, Storeng R, Scudiero D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* (Bethesda) 1990;82:1107–12.
- Tallarida RJ. Drug synergism: its detection and applications. *J Pharmacol Exp Ther* 2001;298:865–72.
- Spanswick VJ, Hartley JM, Ward TH, Hartley JA. Measurement of drug-induced interstrand crosslinking using single-cell gel electrophoresis (Comet) assay. In: Brown R, Boger-Brown U, editors. *Methods in molecular medicine*. Vol 28. Cytotoxic drug resistance mechanisms. Totowa, NJ: Humana Press; 1999:143–54.
- Hartley JM, Spanswick VJ, Gander M, et al. Measurement of DNA cross-linking in patients on ifosfamide therapy using the single cell gel electrophoresis (comet) assay. *Clin Cancer Res* 1999;5:507–12.
- Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumour and normal cells measured using the “comet” assay. *Radiat Res* 1990;122:86–94.
- Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000;6:4885–92.
- Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 (“Iressa”) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001;61:7184–8.

19. Zwelling LA, Anderson T, Kohn KW. DNA-protein and DNA interstrand cross-linking by cis- and trans-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity. *Cancer Res* 1979;39:365–9.
20. Boulton S, Kyle S, Durkacz BW. Mechanisms of enhancement of cytotoxicity in etoposide and ionising radiation-treated cells by the protein kinase inhibitor wortmannin. *Eur J Cancer* 2000;36:535–41.
21. Xu W, Liu L, Smith GC, Charles G. Nitric oxide upregulates expression of DNA-PKcs to protect cells from DNA-damaging anti-tumour agents. *Nat Cell Biol*. 2000; 2:339–45.
22. Benhar M, Engelberg D, Levitzki A. Cisplatin-induced activation of the EGF receptor. *Oncogene* 2002;21:8723–31.
23. Bandyopadhyay D, Mandal M, Adam L, Mendelsohn J, Kumar R. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. *J Biol Chem* 1998; 273:1568–73.
24. Huang SM, Li J, Armstrong EA, Harari PM. Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa) *Cancer Res* 2002;62: 4300–6.
25. Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G. 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 *Clin Cancer Res* 2003;9:2316–26.
26. Critchlow SE, Jackson SP. DNA end-joining: from yeast to man. *Trends Biochem Sci* 1998;23:394–8.
27. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature* 2001;411:366–74.
28. Adachi N, Suzuki H, Iizumi S, Koyama H. Hypersensitivity of nonhomologous DNA end-joining mutants to VP-16 and ICRF-193: implications for the repair of topoisomerase II-mediated DNA damage. *J Biol Chem* 2003;278:35897–902.
29. Durant S, Karran P. Vanillins: a novel family of DNA-PK inhibitors. *Nucleic Acids Res* 2003;31:5501–12.
30. Wang ZM, Chen ZP, Xu ZY, et al. In vitro evidence for homologous recombinational repair in resistance to melphalan. *J Natl Cancer Inst* (Bethesda) 2001;93:1473–8.
31. Panasci L, Xu ZY, Bello V, Aloyz R. The role of DNA repair in nitrogen mustard drug resistance. *Anticancer Drugs* 2002;13:211–20.
32. De Silva IU, McHugh PJ, Clingen P, Hartley JA. Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol Cell Biol* 2000;20: 7980–90.
33. McHugh PJ, Clingen P, Hartley JA, Inusha U, De Silva P. Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. *Nucleic Acids Res* 2002;30:3848–56.
34. Huang S-M, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* 2000;6:2166–74.
35. Magne N, Fischel JL, Tiffon C, et al. Molecular mechanisms underlying the interaction between ZD1839 ('Iressa') and cisplatin/5-fluorouracil. *Br J Cancer* 2003;8:585–92.
36. Xu JM, Azzariti A, Severino M, Lu B, Colucci G, Paradiso A. Characterization of sequence-dependent synergy between ZD1839 ('Iressa') and oxaliplatin. *Biochem Pharmacol* 2003;66:551–63.
37. Magne N, Fischel JL, Dubreuil A, et al. ZD1839 (Iressa) modifies the activity of key enzymes linked to fluoropyrimidine activity: rational basis for a new combination therapy with capecitabine. *Clin Cancer Res* 2003;9:4735–42.