Epidermal Growth Factor Receptor Activity Determines Response of Colorectal Cancer Cells to Gefitinib Alone and in Combination with Chemotherapy

Sandra Van Schaeybroeck,1,2 Anthi Karaiskou-McCaul,1 Donal Kelly,1 Daniel Longley,1 Leeona Galligan,1 Eric Van Cutsem,2 and Patrick Johnston1

Abstract

Purpose: Up to now, there have been no established predictive markers for response to epidermal growth factor receptor (EGFR/HER1/erbB1) inhibitors alone and in combination with chemotherapy in colorectal cancer. To identify markers that predict response to EGFR-based chemotherapy regimens, we analyzed the response of human colorectal cancer cell lines to the EGFR-tyrosine kinase inhibitor, gefitinib (Iressa, AstraZeneca, Wilmington, DE), as a single agent and in combination with oxaliplatin and 5-fluorouracil (5-FU).

Experimental Design: Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and crystal violet cell viability assays and analyzed by ANOVA. Apoptosis was measured by flow cytometry, poly(ADP-ribose) polymerase, and caspase 3 cleavage. EGFR protein phosphorylation was detected by Western blotting.

Results: Cell lines displaying high constitutive EGFR phosphorylation (a surrogate marker for EGFR activity) were more sensitive to gefitinib. Furthermore, in cell lines exhibiting low constitutive EGFR phosphorylation, an antagonistic interaction between gefitinib and oxaliplatin was observed, whereas in cell lines with high basal EGFR phosphorylation, the interaction was synergistic. In addition, oxaliplatin treatment increased EGFR phosphorylation in those cell lines in which oxaliplatin and gefitinib were synergistic but down-regulated EGFR phosphorylation in those lines in which oxaliplatin and gefitinib were antagonistic. In contrast to oxaliplatin, 5-FU treatment increased EGFR phosphorylation in all cell lines and this correlated with synergistic decreases in cell viability when 5-FU was combined with gefitinib.

Conclusions: These results suggest that phospho-EGFR levels determine the sensitivity of colorectal cancer cells to gefitinib alone and that chemotherapy-mediated changes in phospho-EGFR levels determine the nature of interaction between gefitinib and chemotherapy.

Colorectal cancer is the second most common cause of cancer death in the United States and Europe. Advances in first-line treatment of metastatic colorectal cancer have been achieved by addition of agents such as the topoisomerase I inhibitor CPT-11 and the DNA-damaging agent oxaliplatin to 5-fluorouracil (5-FU) – based chemotherapy with response rates in the 40% to 50% range (1–4). Despite these improvements, the overall clinical effect of these therapies remains modest with the median survival times of 20 months (1–4). The generation of selective agents, targeting malignant angiogenesis (5), cell cycle regulation (6), and transduction of growth stimulatory signals (7), has heralded an era of new therapeutic opportunities.

Epidermal growth factor receptor (EGFR) is a member of the HER subfamily of four closely related receptors: EGFR (erbB1/HER1), ErbB2 (HER-2/neu), ErbB3 (HER3), and ErbB4 (HER4). The receptor is a 170-kDa transmembrane glycoprotein, with an extracellular ligand-binding domain and an intracellular region containing the tyrosine kinase domain (8). Ligand binding induces homodimerization or heterodimerization with members of the HER subfamily, resulting in protein tyrosine kinase activation and autophosphorylation of specific tyrosine residues at the COOH terminus. These phosphorylated tyrosines provide docking sites for various Src homology 2 and phosphotyrosine binding domain-containing signaling molecules (9). The two major signaling pathways regulated by EGFR are the Ras/Raf/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways (10).

EGFR and its cognate ligands are frequently overexpressed in different types of solid tumors, including colorectal cancer (11). High EGFR expression has been associated with resistance to standard therapies (12) and has been found to act as a strong prognostic indicator in head and neck, ovarian, cervical,
bladder, and esophageal cancer (13, 14). However, EGFR protein expression has not been found to be a prognostic marker in colorectal cancer (15).

Several strategies have been developed for targeting the EGFR, including monoclonal antibodies that block the extracellular ligand-binding domain of the receptor, recombinant proteins containing transforming growth factor-α or EGFR fused to toxins, low molecular weight tyrosine kinase inhibitors (TKI), and antisense oligonucleotides to the EGFR mRNA (16). The two most promising and clinically developed strategies are the administration of specific anti-EGFR monoclonal antibodies (e.g., IMC-C225 and ABX-EGF) and low molecular weight EGFR-specific TKI (e.g., gefitinib and erlotinib; refs. 10, 16).

Gefitinib (Iressa, ZD-1839, AstraZeneca, Wilmington, DE) is a synthetic anilinoquinazoline and orally active selective EGFR-TKI that acts as a competitive inhibitor of ATP for binding to the EGFR tyrosine kinase pocket and a noncompetitive inhibitor of peptide binding to EGFR (17, 18). Several studies have shown that gefitinib blocks EGF/transforming growth factor-α-stimulated EGFR phosphorylation in a dose-dependent and complete manner (17, 19). The drug inhibits the proliferation of several cancer cell lines in vitro and has antitumor activity in a wide range of xenografts (19–21). Recently, some in vitro and animal studies have shown that coadministration of chemotherapeutic drugs with gefitinib enhances their cytotoxic effect (22–25). In phase I studies, gefitinib has shown activity as a single agent in patients with various solid tumors (26). Two large phase II trials (IDEAL-1 and IDEAL-2) confirmed modest antitumor activity of gefitinib as second-line and third-line treatment of patients with advanced non–small cell lung carcinoma (NSCLC; refs. 27, 28). Two recent publications showed that the presence of EGFR “gain-of-function” mutations in tumors of patients with NSCLC corresponded with sensitivity to gefitinib (29, 30).

The aim of the present study was to analyze the molecular characteristics that determine response to gefitinib alone, or in combination with chemotherapy, in a panel of human colorectal cancer cell lines.

**Materials and Methods**

**Materials.** All chemicals and reagents of Analar grade were obtained from BDH Laboratory Supplies (Poole, England), unless otherwise stated. Gefitinib was provided by AstraZeneca (Macclesfield, United Kingdom). A 10 mmol/L working solution in DMSO was prepared and stored at −20°C. Oxaliplatin was purchased from Sanofi-Synthelabo (Alnwick, United Kingdom). A 1 mmol/L stock solution was prepared in injection water (Braun, Irvine, CA) and stored at 4°C. 5-FU was obtained from Sigma Chemical Co. (St. Louis, MO). A 1 mmol/L stock solution in 1× PBS was prepared and stored at 4°C.

**Cell culture.** All tissue culture reagents were obtained from Invitrogen (Paisley, Scotland), unless otherwise stated. HCT116-p53+/+ and HCT116-p53−/− isogenic human colorectal cancer cell lines were kindly provided by Bert Vogelstein (John Hopkins University, Baltimore, MD) and maintained in McCoy’s 5A medium. RKO and H630 colorectal cancer cells, provided by Bert Vogelstein (John Hopkins University, Baltimore, MD) and maintained in McCoy’s 5A medium. A431 squamous carcinoma cell line, provided by Cancer Research UK, was stored at 4°C. Culture medium was replaced by drug-free medium and cells were allowed to grow for 3 days. Cells were fixed with methanol and stained with 0.01% crystal violet for 5 minutes at room temperature. Stained cells were reabsorbed in ethanol supplemented with 0.2 mol/L sodium citrate (1:1 dilution). Viability was determined by measuring the absorbance at 570 nm. Experimental conditions were tested in quintuplicate, and separate experiments were done on at least three separate occasions.

**Crystal violet cell viability assay.** Cells were seeded at 1,000 to 4,000 per well in 12-well plates. Twenty-four hours after seeding, triplicate wells were exposed to gefitinib and oxaliplatin in different sequences as described above. Five days after cell seeding, culture medium was replaced by drug-free medium and cells were allowed to grow for 3 days. Cells were fixed with methanol and stained with 0.01% crystal violet for 5 minutes at room temperature. Stained cells were reabsorbed in ethanol supplemented with 0.2 mol/L sodium citrate (1:1 dilution). Viability was determined by measuring the absorbance at 570 nm. Experimental conditions were tested in quintuplicate and separate experiments were done at least in duplicate.

**Flow cytometric analysis and cell death measurement.** Cells were seeded at a density of 5 × 10^4 or 1 × 10^5 per well in six-well plates. After 24 hours, duplicate wells were cotreated with gefitinib and oxaliplatin. DNA content was evaluated by propidium iodide (Sigma) staining of cells by resuspending them in 360 μL of 1 mg/mL propidium iodide, 100 mg/mL RNase, and 1× PBS/0.1% FCS. Samples were analyzed using the EPICS XL Flow Cytometer (Couler, Miami, FL) and the extent of death was determined by measuring the sub-G0-G1 population.

**Detection of cell surface epidermal growth factor receptor expression.** Cells were seeded at a density of 1.5 × 10^5 per well in six-well plates or 2 × 10^4 cells in a P140 plate. Twenty-four hours after seeding, cells were treated with increasing doses of oxaliplatin or 5-FU for 24 hours. Cells were incubated with anti-EGFR monoclonal antibody (1:50; SC-120, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at 4°C. A mouse IgG1 (DAKO, Santa Barbara, CA) was used as isotype-matched control. For detection, FITC-labeled goat anti-mouse IgG antibody (Sigma) was used. Fluorescence was evaluated using the EPICS XL Flow Cytometer (Couler). Western blotting. Cells were harvested in ice-cold PBS, pelleted, and snap-frozen in liquid N2. Cell pellets were resuspended in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1%NP40, 0.5% sodium deoxycholate, 0.1% SDS] with protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), 1 mmol/L sodium orthovanadate (Sigma), and 10 mmol/L sodium fluoride. Cells were then lysed by passing them through a 25-gauge needle and centrifuged at 16,180 rcf/4°C for 20 minutes to remove cell debris. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Reagent (Pierce, Rockford, IL). Ten to 40 μg of each protein sample were resolved on SDS-polyacrylamide gels (8-12%) and transferred to a polyvinylidene difluoride Hybond-P membrane (Amersham, Arlington Heights, IL). Immunodetections were done using anti-poly(ADP-ribose) polymerase.
(PARP; C2-10, BD Biosciences Pharmingen, San Diego, CA), anti-EGFR (clone 13, BD Biosciences Pharmingen), and anti-HER2 (clone e2-4001, Neomarkers/Lab Vision Corp., Freemont, CA) mouse monoclonal antibodies in conjunction with a horseradish peroxidase– conjugated anti-mouse secondary antibody (Amersham). Anti-EGFR (sc-O3, Santa Cruz Biotechnology), anti-phospho-EGFR (Tyr1068, Calbiochem, La Jolla, CA), anti-phospho-neu (Tyr1244, sc-12352-R, Santa Cruz Biotechnology), and anti-cleaved caspase 3 (Asp175, Cell Signaling Technology, Beverly, MA) rabbit polyclonal antibodies were used in conjunction with an horseradish peroxidase– conjugated anti-rabbit secondary antibody (Amersham). The Super Signal chemiluminescent system (Pierce) or enhanced chemiluminescence-plus (Amersham) was used for detection.

Reverse transcription-PCR and nucleotide sequence analysis. Total RNA from the various human cancer cell lines was isolated using the RNA STAT-60 reagent (Biogenesis, Poole, United Kingdom) according to the manufacturer’s instructions. Reverse transcription was carried out with 1 µg of RNA using 1 µL of Moloney murine leukemia virus reverse transcriptase (200 units/µL) and 1 µL of random primers (0.3 µg/µL), according to the manufacturer’s instructions. Exons 17 to 23 of the EGFR gene were amplified by PCR, using a forward (5'-GGCTGTCAAGGACTCT-3') and reverse (5'-CATCCTGTGATATGCTTGGAT-3') primer set. PCR products were purified using Wizard PCR clean-up system (Promega, Southampton, United Kingdom) before sequencing. Each sequencing reaction contained 20 ng of template and 3.2 µL of primer (2 µmol/L). Two forward primers (5'-CATGCGAAGGCGCCACAT-3' in exon 17 and 5'-AGGTCCTCAACAAGACTCT-3' in exon 18) and two reverse primers (5'-TCCGTCTATGGCTTGGAT-3' in exon 23 and 5'-CACCGATGCAGCACGTAC-3' in exon 20) were used. The samples were analyzed on an ABI 3730X1 automatic DNA sequencer (AraDx, Craigavon, Northern Ireland).

Quantitative real-time PCR. RNA extraction and reverse transcription was carried out as described above. Real-time PCR was done using the Opticon 2 DNA engine (MJ Research, San Francisco, CA) with a 10-µL mastermix containing SYBR green (DyNamo SYBR Green qPCR kit, Finnzyme Co., Espoo, Finland); forward (5'-AGGACCAAGCAACTGATGCA-3') and reverse (5'-CTTCCAACCCAGTCTTCTC-3') primers at a final concentration of 0.4 µmol/L and 1 µL of cDNA. For this primer set, the expected size of the PCR product was confirmed by agarose gel electrophoresis. The thermal cycling conditions were initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. As an endogenous control, 18S rRNA was amplified in each cycle of 95°C for 1 minute. As an endogenous control, 18S rRNA was amplified in each cycle. Real-time PCR was done using the Opticon 2 DNA engine (MJ Research, San Francisco, CA) with a 10-µL mastermix containing SYBR green (DyNamo SYBR Green qPCR kit, Finnzyme Co., Espoo, Finland); forward (5'-AGGACCAAGCAACTGATGCA-3') and reverse (5'-CTTCCAACCCAGTCTTCTC-3') primers at a final concentration of 0.4 µmol/L and 1 µL of cDNA. For this primer set, the expected size of the PCR product was confirmed by agarose gel electrophoresis. The thermal cycling conditions were initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. As an endogenous control, 18S rRNA was amplified in each cycle. Real-time PCR was done with a final concentration of 5 µmol/L cDNA. The thermal cycling conditions were initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. As an endogenous control, 18S rRNA was amplified in each cycle.

Drug interaction analysis. The nature of the interaction between gefitinib and oxaliplatin or 5-FU was determined by calculating the combination index (CI) according to the method of Chou and Talalay (33). CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively. CI values were calculated from isobolograms generated using the CalcuSyn software programme (BioSoft, Cambridge, UK).

Statistical analysis. To further assess the statistical significance of the interactions, we designed a univariate ANOVA analysis using the SPSS software package. This was an additive model based on the null hypothesis that there was no interaction between the drugs.

**Results**

Epidermal growth factor receptor and HER2 expression and activation in colorectal cancer cells. Although EGFR is frequently overexpressed in solid tumors including colorectal cancer, several studies have failed to establish a clear relationship between EGFR expression level and sensitivity to gefitinib. We compared the basal protein expression level of EGFR in colorectal cancer cells by immunoblotting (Fig. 1A) and flow cytometry (Fig. 1B). As an internal control, total protein and cell surface expression in the A431 cell line were determined (Fig. 1A and B). EGFR was expressed in all cell lines and was slightly higher in the HCT116-p53+/− and HCT116-p53−/− cell lines compared with the LoVo cell line and significantly higher compared with the RKO and H630 cell lines. In addition, EGFR gene expression was determined by quantitative real-time reverse transcription-PCR. Among the colorectal cancer cell lines, EGFR mRNA levels were similar, with slightly higher expression in the HCT116-p53+/− and HCT116-p53−/− cell lines (Fig. 1C). EGFR mRNA expression in the A431 cell line was very high, which is in accordance with the known EGFR gene amplification in this cell line (34). HER2 is the preferred dimerization partner for EGFR, resulting in a heterodimer that exhibits an increased rate of stability and signaling potency compared with homodimers (35). Furthermore, it has been reported that gefitinib is a potent inhibitor of growth of HER2-overexpressing cancer cells (36). Therefore, we determined the basal HER2 expression level in all five colorectal cancer cell lines and found that HER2 cellular content was similar for all the colorectal cancer cell lines (Fig. 1A). We then analyzed the tyrosine phosphorylation status of EGFR in unstimulated cells (basal phosphorylation level) and EGF-stimulated cells, using a phosphospecific EGFR antibody (directed against phospho-Y1068 and reflecting the activation state of the receptor; ref. 37). The LoVo and H630 cell lines, along with the A431 cells, showed high basal EGFR phosphorylation compared with the HCT116-p53+/−, HCT116-p53−/−, and RKO cell lines, indicating that basal levels of EGFR and phospho-EGFR do not necessarily correlate (Fig. 1A). The basal HER2 phosphorylation level was also determined, using an antibody which recognizes the phospho-Y1248 residue. Phospho-HER2 levels were similar in all five colorectal cancer cell lines, although it was slightly higher in the H630 cell line (Fig. 1A). Stimulation with 100 ng/mL EGF increased the level of phospho-EGFR in all five colorectal cancer cell lines (Fig. 1D). However, following stimulation with EGF, a significant increase of the level of phospho-HER2 was only observed in the LoVo cell line (Fig. 1D). It is well established that ligand binding promotes not only tyrosine phosphorylation of the EGFR but also its rapid internalization (38). In all five colorectal cancer cell lines, reduced EGFR receptor cell surface expression was observed following EGF (100 ng/mL) stimulation, suggesting ligand-induced receptor internalization (data not shown). We further determined the effect of gefitinib on EGF-stimulated EGFR and HER2 phosphorylation and EGFR internalization. Treatment with 10 µmol/L gefitinib for 24 hours inhibited EGF-induced EGFR phosphorylation levels in all five cell lines; however, a decrease in phospho-HER2 levels was only observed in LoVo, H630, and A431 cell lines (Fig. 1D; data not shown). Gefitinib did not affect cell surface expression of EGFR nor its internalization following stimulation with EGF (data not shown).

Sensitivity to gefitinib alone correlates with basal phosphorylation status of epidermal growth factor receptor. We assessed the antiproliferative activity [IC50 (72 hours) values] of gefitinib for the different cell lines using an in vitro growth-inhibitory assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylyltetrazolium bromide; Fig. 1A]. The LoVo, H630, and A431 cell lines were the...
most sensitive to gefitinib (with IC_{50} doses of 2.7, 1.9, and 1.2 \mu M, respectively). The HCT116 and RKO cell lines were less sensitive to gefitinib, with 3- to 4-fold higher IC_{50} (72 hours) doses. Comparison of the IC_{50} (72 hours) values with our immunoblotting results indicated that there was a clear association between basal EGFR phosphorylation level and sensitivity to gefitinib, as cancer cells exhibiting low basal EGFR phosphorylation were more resistant to gefitinib (Fig. 1A). However, total EGFR protein and mRNA expression did not correlate with the growth-inhibitory effect of gefitinib in these cell lines (Fig. 1A and C). Furthermore, there was no clear correlation between p53 status and sensitivity to gefitinib (Fig. 1A).

To determine whether there was a correlation between basal EGFR phosphorylation and the presence of mutations in the receptor tyrosine kinase domains, we sequenced exons 17 to 23 in the five colorectal cancer cell lines, thereby searching for specific "activating" mutations recently described in patients with NSCLC (29, 30). Screening our five colorectal cancer cell lines and the A431 cell line for these mutations did not reveal any deletions (e.g., deletion at codons 746-753 in exon 19) or missense mutations (e.g., L858R and L861Q in exon 21 and G719C in exon 18; data not shown). These data suggest that in our subgroup of cancer cells, EGFR phosphorylation level cannot be linked to the presence of activating mutations within the tyrosine kinase domain.

Evaluation of the antiproliferative activity of gefitinib in combination with oxaliplatin. We next examined the growth-inhibitory effects of gefitinib in combination with the chemotherapeutic agent oxaliplatin. Colorectal cancer cells and the A431 cell line were exposed to gefitinib and oxaliplatin in three schedules as described in Materials and Methods. Significant antagonism was observed between gefitinib- and oxaliplatin-induced growth inhibition in the HCT116-p53^{+/+}, HCT116-p53^{--/}, and RKO cell lines, with the
interaction was observed in HCT116-p53+/+ and HCT116-
synergistic effect. Furthermore, as the same antagonistic
combination of drugs had an additive or supra-additive/
lines with high basal EGFR phosphorylation, the same
gefitinib resulted in significant antagonism, whereas in cell
phosphorylation levels, the combination of oxaliplatin with
clearly indicate that in cell lines with low basal EGFR
observed in H630 and LoVo cells (Fig. 2B). The above results
inhibitory effect between gefitinib and oxaliplatin was
In contrast, an additive or supra-additive/synergistic growth-
and RKO cells, particularly for sequences I and II (Fig. 2B).
In contrast with the above results, the combination of oxaliplatin
resulted in significant decreases in the apoptotic population
resulted in significant decreases in the apoptotic population
compared with cells treated with oxaliplatin alone (Fig. 3A, bottom).
We further determined the effect of gefitinib on oxaliplatin-
induced apoptosis by examining PARP cleavage, a well-
established molecular marker of the onset of apoptosis (40).
In HCT116-p53+/+ cells, treatment with 0.5 and 1 µmol/L
oxaliplatin induced PARP cleavage. However, coadministration
of gefitinib (1 and 2.5 µmol/L) with oxaliplatin resulted in
a dose-dependent inhibition of oxaliplatin-induced PARP
cleavage in these cells (Fig. 3B). Similar results were obtained
in the RKO cell line (data not shown). In the HCT116-p53+/+
cell line, although we were unable to detect any PARP
cleavage in response to oxaliplatin, treatment with 5 and 7.5
µmol/L oxaliplatin induced caspase 3 cleavage, which was
inhibited by coadministration of 1 and 2.5 µmol/L gefitinib
(Fig. 3B). In LoVo cells, treatment with 1 and 3 µmol/L
oxaliplatin induced PARP cleavage, and this was increased
following coadministration of 0.2 and 0.4 µmol/L gefitinib

Effect of gefitinib on oxaliplatin-induced apoptosis. Flow
cytometry was used to examine the level of apoptosis in the
panel of colorectal cancer cell lines, following treatment
with gefitinib and oxaliplatin. Treatment with gefitinib has
been shown to induce blockade of cell cycle progression at the
G1-S phase boundary (39). In accordance with this, we found a
shift to the G1-S boundary following gefitinib treatment in all
five colorectal cancer cell lines (data not shown). This
was accompanied by no or only a minor increase in the apoptotic
sub-G0-G1 phase (Fig. 3A). In HCT116-p53+/+, HCT116-p53−/−,
and RKO cell lines, cotreatment with gefitinib and oxaliplatin
resulted in significant decreases in the apoptotic population
compared with cells treated with oxaliplatin alone (Fig. 3A, top). The same observations were obtained with all three
treatment schedules (data not shown). In contrast, when H630
and LoVo cells were exposed to cotreatment with oxaliplatin
and gefitinib, an increase in apoptosis was observed compared
with cells treated with oxaliplatin alone (Fig. 3A, bottom).

We further determined the effect of gefitinib on oxaliplatin-
induced apoptosis by examining PARP cleavage, a well-
established molecular marker of the onset of apoptosis (40).
In HCT116-p53+/+ cells, treatment with 0.5 and 1 µmol/L
oxaliplatin induced PARP cleavage. However, coadministration
of gefitinib (1 and 2.5 µmol/L) with oxaliplatin resulted in
a dose-dependent inhibition of oxaliplatin-induced PARP
cleavage in these cells (Fig. 3B). Similar results were obtained
in the RKO cell line (data not shown). In the HCT116-p53+/+
cell line, although we were unable to detect any PARP
cleavage in response to oxaliplatin, treatment with 5 and 7.5
µmol/L oxaliplatin induced caspase 3 cleavage, which was
inhibited by coadministration of 1 and 2.5 µmol/L gefitinib
(Fig. 3B). In LoVo cells, treatment with 1 and 3 µmol/L
oxaliplatin induced PARP cleavage, and this was increased
following coadministration of 0.2 and 0.4 µmol/L gefitinib

majority of CI values >1 for each schedule (Fig. 2A, top; data
not shown). This antagonism was more pronounced following
concomitant treatment and pretreatment with gefitinib. In
contrast with the above results, the combination of oxaliplatin
with gefitinib induced additive/synergistic growth-inhibitory
effects (CI < 1) at most concentrations in the LoVo and H630
colorectal and A431 cell lines, in all three sequences (Fig. 2A, bottom; data not shown).

Similar results were obtained from crystal violet cell viability
assays, which showed significant antagonism between gefitinib
and oxaliplatin in HCT116-p53+/+, HCT116-p53−/−,
and RKO cells, particularly for sequences I and II (Fig. 2B).
In contrast, an additive or supra-additive/synergistic growth-
inhibitory effect between gefitinib and oxaliplatin was
observed in H630 and LoVo cells (Fig. 2B). The above results
clearly indicate that in cell lines with low basal EGFR
phosphorylation levels, the combination of oxaliplatin with
gefitinib resulted in significant antagonism, whereas in cell
lines with high basal EGFR phosphorylation, the same
combination of drugs had an additive or supra-additive/
synergistic effect. Furthermore, as the same antagonistic
interaction was observed in HCT116-p53+/+ and HCT116-
p53−/− cell lines, our data suggest that p53 does not play a
role in regulating this interaction.

Fig. 2. Cell viability assays in colorectal cancer cell lines in response to gefitinib and oxaliplatin. Cells were treated with no
drug (control), gefitinib alone, oxaliplatin alone, or gefitinib in combination
with oxaliplatin. To evaluate the interaction between gefitinib and oxaliplatin, we used
the method of Chou and Talalay (CI value),
where CI < 1, CI = 1, and CI > 1 denotes,
respectively, synergism, an additive
interaction, and antagonism. A. CI values
obtained from 3-(4,5-dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide
assays in cell lines with low basal EGFR
phosphorylation (top) and high basal
EGFR phosphorylation (bottom), treated
concomitantly with gefitinib and oxaliplatin
for 96 hours. One representative
experiment is shown (experiment carried
out at least thrice for each cell line). CI value
for the combination of 2.5 µmol/L gefitinib
and 1 µmol/L oxaliplatin in HCT116-p53−/−
cells fell beyond the scale of the graph.
B. CI values obtained from crystal
violet cell viability assays in colorectal
cancer cell lines treated with gefitinib
and oxaliplatin for the different schedules.
A similar increase in PARP cleavage following combination of oxaliplatin with gefitinib was observed in the A431 cell line (Fig. 3B). Despite the increased apoptosis observed by cell cycle analyses in H630 cells, we were unable to detect PARP or caspase 3 cleavage following oxaliplatin or gefitinib/oxaliplatin treatment in these cells.

Collectively, these results indicate that gefitinib antagonizes oxaliplatin-induced apoptosis in cell lines with low basal EGFR phosphorylation and has the opposite effect in cell lines with high basal EGFR phosphorylation.

**Effect of oxaliplatin on epidermal growth factor receptor phosphorylation.** To begin to elucidate the mechanisms involved in regulating the interaction between oxaliplatin and gefitinib, we examined the effect of oxaliplatin on EGFR phosphorylation in the five colorectal cancer cells and A431 cell line. In HCT116-p53+/+, HCT116-p53−/−, and RKO cell lines, a dose-dependent decrease in phospho-EGFR levels was observed 24 hours after oxaliplatin exposure (Fig. 4A). In contrast, in H630, LoVo, and A431 cells, oxaliplatin exposure resulted in a dose-dependent increase in EGFR phosphorylation (Fig. 4B). Interestingly, in the HCT116-p53+/+ cell line, the decrease in phospho-EGFR levels was observed at concentrations well below the IC50 dose, whereas in the HCT116-p53−/− and RKO cell lines, the decrease was only observed at concentrations equal to and above the IC50 dose. In the LoVo, H630, and A431 cell lines, enhanced phospho-EGFR levels was observed at concentrations below the IC50 dose. There was no change in total and EGFR cell surface expression following treatment with oxaliplatin (Fig. 4A-C). These results indicate that oxaliplatin treatment up-regulates phospho-EGFR in those cell lines in which gefitinib enhances the effect of the drug but has the opposite effect on phospho-EGFR in cell lines in which gefitinib and oxaliplatin are antagonistic.
Evaluation of the antiproliferative activity of gefitinib in combination with 5-fluorouracil. As oxaliplatin is used in combination with the antimetabolite 5-FU in the treatment of colorectal cancer (FOLFOX), we also examined the interaction between gefitinib and 5-FU. HCT116-p53+/+, HCT116-p53−/−, RKO, H630, and LoVo cell lines were exposed to gefitinib and 5-FU in the three schedules as described above. In all of these cell lines, a supra-additive/synergistic interaction between the two drugs was observed for all three sequences (Fig. 5; data not shown). Furthermore, in each cell line, a dose-dependent increase in EGFR activation, which was not accompanied by an increase in total and cell surface EGFR expression, was observed following 5-FU exposure (Fig. 5B and C). Thus, the supra-additive/synergistic interaction between 5-FU and gefitinib in these cell lines correlated with an increase in phospho-EGFR levels in response to 5-FU treatment. In addition, similar results were obtained with the active metabolite of CPT-11, SN-38, which induced EGFR phosphorylation and interacted synergistically with gefitinib in each cell line to reduce cell viability (data not shown).

**Discussion**

Previous *in vitro* and *in vivo* studies have shown that EGFR-targeted TKIs such as gefitinib (Iressa) and erlotinib (Tarceva) enhance the growth inhibitory effects of a number of cytotoxic drugs, which differ in their mechanism of action (19, 21–25, 41, 42). In these studies, no correlation was found between EGFR expression level and drug interaction. In the present study, we investigated the cytotoxic effect of gefitinib in combination with oxaliplatin or 5-FU in five human colorectal cancer cell lines, together with the A431 cell line, each of which express different basal levels of phospho-EGFR.

Initially, we examined the sensitivity of these cancer cell lines to gefitinib as a single agent. We found that neither EGFR expression level nor p53 status predicted sensitivity of cancer cells to gefitinib alone, in agreement with several previous studies (17, 21, 22, 43, 44). In contrast, we found that sensitivity to gefitinib alone was determined by the constitutive level of EGFR phosphorylation. Cell lines with high basal EGFR phosphorylation (H630, LoVo, and A431) were more sensitive to gefitinib treatment compared with cell lines with low basal EGFR phosphorylation (HCT116-p53+/+, HCT116-p53−/−, and RKO), a result which may be of high clinical relevance. Interestingly, two recent publications showed that the presence of EGFR mutations in tumors of patients with NSCLC corresponded with sensitivity to gefitinib (29, 30). These gain-of-function mutations reside in exons 18 to 21, are clustered near the ATP cleft of the tyrosine kinase domain, and are linked...
with increased receptor activation after ligand binding and enhanced receptor dephosphorylation induced by gefitinib. However, screening of our cell lines for these mutations did not reveal any deletions or missense mutations (data not shown). In accordance with our findings, a recent study did not observe any EGFR mutations in solid tumors (including colorectal cancers) except for non–small cell lung cancers (45). Nevertheless, other mechanisms, such as autocrine growth factor loops, heterodimerization within the HER subfamily, or cross-talk with heterologous receptors, could be responsible for increased constitutive EGFR phosphorylation (46). Indeed, in the H630, LoVo, and A431 cells, we found significantly decreased levels of HER2 phosphorylation following treatment with gefitinib, suggesting an interaction between EGFR and HER2, which could account for the high basal EGFR phosphorylation and increased sensitivity to gefitinib of these cells.

We also evaluated the effect of combined treatment with gefitinib and chemotherapy. We observed a significant antagonism between gefitinib and oxaliplatin-induced cell death in cell lines with low basal EGFR phosphorylation levels, whereas in cell lines with high basal EGFR phosphorylation, the interaction between gefitinib and oxaliplatin was additive and often supra-additive/synergistic. These results indicate that constitutive phospho-EGFR levels may be just as important in predicting response to gefitinib/oxaliplatin cotreatment as to gefitinib monotherapy. To elucidate the biochemical mechanism underlying the interaction between gefitinib and oxaliplatin, we analyzed the effect of oxaliplatin on EGFR phosphorylation. Interestingly, the interactions between gefitinib and oxaliplatin correlated with the effect of oxaliplatin on EGFR phosphorylation. In cell lines in which oxaliplatin and gefitinib interacted antagonistically, a dose-dependent decrease
in phosphorylation was observed following oxaliplatin treatment, whereas in cell lines in which oxaliplatin and gefitinib interacted synergistically, oxaliplatin exposure resulted in increased EGFR phosphorylation. We also investigated the interaction between 5-FU and gefitinib in these cell lines. In contrast to our data with oxaliplatin, the interaction between 5-FU and gefitinib was supra-additive/synergistic in each cell line and so did not correlate with initial basal phospho-EGFR levels. However, in accordance with our oxaliplatin results, the synergistic interaction between 5-FU and gefitinib did correlate with increased EGFR activation in each cell line following drug treatment. Similar observations were made when gefitinib was combined with the topoisomerase I inhibitor SN-38 (the active metabolite of CPT-11): increased EGFR activation following SN-38 correlated with a synergistic interaction between gefitinib and SN-38 in all colorectal cancer cell lines (data not shown). Because EGFR phosphorylation is a major determinant of the sensitivity of these colorectal cancer cells to gefitinib-induced growth inhibition (Fig. 1A), biochemical modulation of phospho-EGFR levels by cytotoxic agents would be expected to determine the nature of the interaction between gefitinib and these drugs. Moreover, similar results were obtained when cisplatin was combined with gefitinib in NSCLC cell lines: only cell lines that responded with increased EGFR phosphorylation following cisplatin were synergistically growth inhibited following the addition of gefitinib to cisplatin. These results could explain in part the negative findings of the INTACT1/INTACT2 trials, in which no preselection of patients likely to benefit from the addition of gefitinib to their chemotherapeutic regimen was made (47, 48). Interestingstly, the mechanism of synergy between gefitinib and chemotherapy seems to be similar in the colorectal cancer and NSCLC cell lines, as both colorectal cancer and NSCLC cells that respond to chemotherapy by up-regulating phospho-EGFR undergo synergistic growth inhibition following the addition of gefitinib to the chemotherapy. In these cell lines, EGFR might be the “Achilles’ heel” on which the cells are dependent for their survival, and increased EGFR activation following chemotherapy renders them more sensitive to EGFR inhibition.

EGFR activation in cancer cells in response to chemotherapy could constitute a prosurvival response. Increased EGFR phosphorylation following exposure to cytotoxic drugs has been shown in other studies (24, 49). Furthermore, a number of reports have suggested that EGFR promotes cell survival and protects cells from death receptor–mediated apoptosis through activation of the mitogen-activated protein kinase or Akt pathways (50). Moreover, gefitinib has been shown to prevent cell survival by blocking the inactivation of the proapoptotic protein BAD (51). p53 mutations, which occur in >50% of patients with colorectal cancer (52), have been shown to play a role in transactivation of the promoter of several growth factors, including EGFR (53), and p53 has been reported to be an important determinant of sensitivity to oxaliplatin and 5-FU (54). However, we observed similar chemotheraphy/gefitinib interactions in isogenic p53 wild-type and null HCT116 sister cell lines (oxaliplatin and gefitinib were antagonistic in both lines, whereas 5-FU and gefitinib were synergistic in both lines). These results suggest that the nature of the interaction (synergistic or antagonistic) between chemotherapy and gefitinib is not dependent on p53 status in colorectal cancer cell line models. In summary, by investigating the sensitivity of a panel of colorectal cancer cell lines to gefitinib alone and in combination with oxaliplatin in a panel of colorectal cancer cell lines, we were able to distinguish two distinct phenotypes: (a) cell lines with low basal EGFR phosphorylation were less sensitive to the growth-inhibitory effects of gefitinib, and the combination of gefitinib with oxaliplatin resulted in significant antagonism; and (b) cell lines with high basal EGFR phosphorylation were more sensitive to gefitinib, and the combination of gefitinib and oxaliplatin resulted in supra-additive inhibitory effects. However, the interaction between 5-FU and gefitinib resulted in only one phenotype, as cell lines with both low and high constitutive EGFR phosphorylation exhibited a synergistic interaction between 5-FU and gefitinib. Furthermore, modulation of EGFR phosphorylation following drug treatment seemed to determine the nature of the interaction between gefitinib and chemotherapy: when drug treatment resulted in increased EGFR activation, a synergistic/supra-additive interaction was observed, whereas when drug treatment resulted in decreased EGFR activation, an antagonistic interaction was observed. These novel data indicate that EGFR phosphorylation levels determine response to gefitinib alone and in combination with chemotherapy. Moreover, our findings suggest that only colorectal cancer tumors that respond to the chemotherapeutic agents 5-FU and oxaliplatin (drugs frequently used in the treatment of colorectal cancer) by up-regulating phospho-EGFR, will respond to the addition of EGFR-targeted TKIs to a 5-FU or platinum-containing regimen. Thus, measurement of phospho-EGFR expression after drug treatment may identify colorectal cancer patients most likely to benefit from addition of an EGFR-targeted TKI to their chemotherapy regimen.

Acknowledgments

We thank AstraZeneca for providing us with the LoVo cells and gefitinib.

References


Epidermal Growth Factor Receptor Activity Determines Response of Colorectal Cancer Cells to Gefitinib Alone and in Combination with Chemotherapy
