Introduction

Non–small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States and Europe (1). Although standard chemotherapy, which consists of a platinum-based regimen, improves survival, disease progression is inevitable (2). For this reason, several new selective agents were introduced for the treatment of this disease (3). The epidermal growth factor (EGF) receptor (EGFR) family is part of a complex signal transduction network that promotes cell growth and survival (4). EGFR overexpression has been observed in 40% to 80% of NSCLC patients, most frequently in the squamous cell and bronchoalveolar carcinoma subtypes (5). Hence, several anti-EGFR agents, including monoclonal antibodies (e.g., cetuximab) and tyrosine kinase inhibitors (TKI; e.g., gefitinib, erlotinib), have been developed and studied in the treatment of NSCLC (6). Gefitinib (Iressa) and erlotinib (Tarceva) are p.o. administered small molecules that compete with ATP binding to the tyrosine kinase pocket and inhibit EGFR activity (7). Two randomized phase II trials evaluated the antitumor activity of gefitinib in patients with NSCLC who had been previously treated with a platinum compound and/or docetaxel (8, 9). These studies (IDEAL-1 and IDEAL-2) reported objective tumor response rates of 18% and 12%, respectively. A similar phase II trial, evaluating the response to erlotinib among previously treated NSCLC patients, showed a response rate of 12.3% (10). Subsequently, in the BR.21 trial, erlotinib was found to improve overall survival for patients with NSCLC who had already been treated with one or two chemotheraphy regimens (11). These encouraging response rates, clinical benefit, and survival advantage led to the introduction of gefitinib and erlotinib in the treatment of patients with locally advanced or metastatic NSCLC, which have been pretreated with chemotherapy (12, 13).
Clinical trials have revealed a significant variability in response to EGFR-TKI. The response to EGFR-TKI was higher in females with adenocarcinomas, nonsmokers, and Asians, whereas males with nonadenocarcinomas, smokers, and Caucasians were less responsive (14–16). The molecular basis for this difference between the two patient groups was elucidated by the discovery of somatic “activating” mutations in the EGFR kinase domain (14–16). These mutations are most frequently small deletions that affect amino acids 746 through 750 (exon 19) or point mutations [most commonly a replacement of leucine by arginine at codon 858 (L858R) in exon 21], although more recently, new single-nucleotide substitutions in exons 18, 20, and 21 were identified (17, 18). The mutations were most frequently identified among females, nonsmokers, and East Asian populations, and are, apart from seven cases in the literature, most commonly associated with adenocarcinomas of the bronchioalveolar subtype (19, 20). These patient characteristics are similar to those that correlate with response to EGFR-targeted TKI. The increased susceptibility of cells carrying mutant receptors may result from a repositioning of critical residues surrounding the ATP-binding cleft, thereby stabilizing their interaction with both ATP and its competitive inhibitors, which leads to enhanced inhibition of the mutant kinase by those inhibitors (21). Moreover, it has been shown that cells carrying the DelE746A-750 and L858R mutations are more dependent on increased survival signals transduced by their mutant receptors, and small interfering RNA–mediated specific inactivation of the mutant receptor resulted in rapid and massive apoptosis (22). Recently, a mutation in exon 20 (T790M) has been associated with primary and secondary resistance to EGFR-targeted TKIs (23–26). Substitution of a threonine at position 790 (which is located in the hydrophobic ATP pocket of the catalytic domain and critical for erlotinib binding to EGFR) by a larger residue, such as methionine, results in a steric hindrance that may interfere with the binding of TKIs (23, 27). Furthermore, there is a suggestion that the clinical benefit observed with anti-EGFR-TKIs is not restricted to those patients harboring EGFR gene mutations (11, 28). In this respect, EGFR protein expression and gene amplification have recently been reported to correlate with response rate (18, 28).

Despite the evidence of single agent activity, the addition of gefitinib or erlotinib to standard chemotherapy (gemcitabine/cisplatin and carboplatin/paclitaxel) in phase III trials in chemonaive patients with advanced NSCLC (INTACT-1 and INTACT-2, TRIBUTE, and TALENT), failed to improve survival, time-to-disease progression, and response rate (29–32). Due to a lack of predictive markers for sensitivity to anti-EGFR agents, no preselection of patients likely to respond to EGFR-TKI was possible. In particular, no predictive markers for response to EGFR-TKIs in combination with standard chemotherapy have been identified. 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 EGFR wild-type and mutant NSCLC cell lines.

Materials and Methods

Materials

All chemicals and reagents of Analar grade were obtained from BDH Laboratory Supplies (Poole, England, United Kingdom) unless otherwise stated. Gefitinib was provided by AstraZeneca (Macclesfield, United Kingdom). A 10 mmol/L working solution of gefitinib in DMSO was prepared and stored at –20°C. Cisplatin was purchased from Mayne Pharma Plc (Warwickshire, United Kingdom). A 10 mmol/L stock solution of cisplatin was prepared in injection water (Hameln Pharmaceuticals, Ltd., Gloucester, United Kingdom) and stored at room temperature. Taxol was obtained from Bristol-Myers Squibb (Middlesex, United Kingdom) and stored at room temperature. Recombinant EGF was purchased from Calbiochem (Darmstadt, Germany) and reconstituted to a concentration of 10 μg/mL in 10 mmol/L acetic acid containing 0.1% bovine serum albumin and stored at –20°C.

Cell Culture

All tissue culture materials were obtained from Invitrogen (Paisley, Scotland, United Kingdom), unless otherwise stated. H23, H157, H460, H727, H838, and H441 human NSCLC cell lines were provided by the National Cancer Institute (Bethesda, MD) and maintained in DMEM and RPMI medium, respectively. H322, Calu6, PC9, H1650, H1975 human NSCLC cell lines, and the A549 and Calu3 NSCLC cell lines, all supplied by AstraZeneca, were grown in RPMI, DMEM, or MEM. All media were supplemented with 2% dialyzed FCS, 50 μg/mL penicillin-streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Invitrogen). All cells were grown in a humidified atmosphere with 5% CO2 at 37°C.

EGFR Sequencing

The H23, H157, H441, H322, H460, H727, Calu3, Calu6, and A549 cells are EGFR wild type (data provided by AstraZeneca and previously published; refs. 14, 33). The H1650 and PC9 cells are known to contain a deletion in exon 19 (DelE746A750) of EGFR, whereas the H1975 cell line carries two missense mutations in EGFR (L858R, T790M; data provided by AstraZeneca).

Cell Viability Assay

Cell viability was assessed by the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma, Dorset, United Kingdom] assay (34). Cells were seeded at 3,200 to 5,000 per well in 96-well plates. Twenty-four hours after seeding, cells were exposed to gefitinib and cisplatin in three sequences: sequence I (concomitant gefitinib and cisplatin), sequence II (pretreatment with gefitinib for 24 hours), and sequence III (pretreatment with cisplatin for 24 hours). For each cell
line, the IC_{30}, IC_{20}, and IC_{10} dose for cisplatin was combined with an IC_{10} and IC_{20} dose for gefitinib. Ninety-six hours after drug treatment, cells were washed once with 1× PBS and incubated with medium containing MTT (0.5 mg/mL in medium) for 3 hours at 37°C. Culture medium with MTT was removed and formazan crystals reabsorbed in 200 μL DMSO (Sigma). Cell viability was determined by measuring the absorbance at 570 nm, using a microplate reader (Molecular Devices, Wokingham, England, United Kingdom). Experimental conditions were tested in quintuplicate and separate experiments were done on at least three separate occasions.

Crystal Violet Assay
Because G_{2}M cell cycle arrest after Taxol treatment may significantly influence mitochondrial number and/or function and, consequently, MTT reduction to formazan (35), we decided to investigate the interaction between gefitinib and Taxol by using the crystal violet assay. Cells were seeded at 3,200 to 3,500 per well in 96-well plates. Twenty-four hours after seeding, cells were exposed to gefitinib and Taxol in different sequences as described above. For each cell line, the IC_{50}, IC_{20}, and IC_{10} dose for Taxol was combined with an IC_{10} and IC_{20} dose for gefitinib. Ninety-six hours after drug treatment, cells were fixed with methanol and stained with 0.01% crystal violet for 5 minutes at room temperature. Stained cells were reabsorbed in a 1:1 mix of 100% ethanol and 0.2 mol/L sodium citrate. 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.

Flow Cytometric Analysis and Cell Death Measurement
Cells were seeded at a density of 1 × 10^{5} per well in six-well plates. After 24 hours, wells were cotreated with gefitinib and either cisplatin or Taxol. DNA content was evaluated by propidium iodide (Sigma) staining of cells (Coulter, Miami, FL) and the extent of cell death was determined by evaluating the percentage of cells with DNA content <2N.

Detection of Cell Surface EGFR Expression
Cells were seeded at a density of 1.5 × 10^{5} per well in six-well plates. Twenty-four hours after seeding, cells were trypsinized and incubated with anti-EGFR monoclonal antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at 4°C. A mouse IgG1 (DAKO, Santa Barbara, CA) was used as an isotype-matched control. Cells were washed thrice with ice-cold PBS and incubated in the dark for 60 minutes at 4°C with FITC-labeled goat anti-mouse IgG secondary antibody (1:20, Sigma). After three washing steps with ice-cold PBS, cells were resuspended in 300 μL 1% paraformaldehyde. Fluorescence was evaluated using the EPICS XL Flow Cytometer (Coulter).

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, and 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 13,200 rpm/4°C for 20 minutes to remove cell debris. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL). Ten to 30 μg of each protein sample were resolved on SDS-polyacrylamide gels (8%) and transferred to a polyvinylidene difluoride Hybond-P membrane (Amersham, Buckinghamshire, United Kingdom). Immunodetections were done using anti-EGFR (clone 13, PharMingen, BD Biosciences, San Jose, CA), anti-Her-2 (clone e2-4001, Neomarkers, Lab Vision Corp., Suffolk, United Kingdom), and anti-phospho-extracellular signal-regulated kinase 1/2 (pErk1/2; Thr202/Tyr204, Cell Signalling, Beverly, MA) mouse monoclonal antibodies in conjunction with a horseradish peroxidase–conjugated anti-mouse secondary antibody (Amersham). Anti-pEGFR (Tyr^{1068}, Calbiochem), anti-pNeu (Tyr^{1248}, Santa Cruz Biotechnology), anti-pAkt (Ser^{473}, Cell Signalling), anti-Akt (Cell signaling), and anti-Erk1/2 (K-23, Santa Cruz) rabbit polyclonal antibodies were used in conjunction with a horseradish peroxidase–conjugated anti-rabbit secondary antibody (Amersham). Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma). The Super Signal chemiluminescent system (Pierce) or ECL–plus (Amersham) were used for detection.

Quantitative Real-time PCR
Total RNA from the various human cancer cell lines was isolated using the RNA STAT-60 reagent (Biogenesis, Poole, United Kingdom) according to the instructions of the manufacturer. Reverse transcription was carried out with 4 μg RNA using 1 μL of Moloney murine leukemia virus reverse transcriptase (200 units/μL) and 1 μL random primers (0.3 μg/μL), according to the instructions of the manufacturer (Invitrogen). 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, Finnzymes, New England Biolabs, Herts, United Kingdom), primers at a final concentration of 0.4 μmol/L, and 1 μL cDNA. As an endogenous control, 185 rRNA was amplified in each sample. The primer sequences used were as follows: EGF forward (5'-AGGACCAAAGCAACATGGCTA-3'); EGF reverse (5'-CCTTGACGCTGTITTTACCT-3'); 18S forward (5'-CATTCTGATTCGCCGCCTGTA-3'); 18S reverse (5'-CGTATCTGATCGTCT-3'). For each primer set, the expected size of the PCR product was confirmed by agarose gel electrophoresis. The following thermal cycling conditions were used for each primer set: initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for

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15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Triplicate samples were analyzed for both EGFR and 18S. For each PCR reaction, a cDNA standard curve was used to generate relative expression changes in EGFR mRNA levels, which were normalized to 18S.

**Statistical Analysis**

The nature of the interaction between gefitinib and cisplatin or Taxol was determined by calculating the combination index (CI) according to the method of Chou and Talalay (36). CI values of <0.3, 0.3 < CI < 0.7, 0.7 < CI < 0.85, 0.85 < CI < 1, 1, and >1 indicate very strong synergism, strong synergism, moderate synergism, slight synergism, an additive interaction, and antagonism, respectively. CI values were calculated from isobolograms generated using the CalcuSyn software program (Microsoft Windows). An unpaired two-tailed t test was used to determine the significance of change in levels of apoptosis between different treatment groups. All changes in levels of apoptosis that are described as significant had P values that were <0.001.

**Results**

**Constitutive Expression Levels and Activity of EGFR, Her2, and Downstream Signaling Molecules in NSCLC Cell Lines and Correlation with Histologic Subtype**

Although EGFR gene mutations were linked with clinical response to EGFR-targeted TKI, there is a suggestion that this clinical benefit is not restricted to patients with these mutations (11, 28). We compared the basal EGFR expression levels of wild-type and mutant NSCLC cells by immunoblotting (Fig. 1A and B) and flow cytometry (Fig. 1C). Total EGFR was expressed in all NSCLC cell lines and generally correlated with cell surface EGFR expression, except for the H460, Calu6, and H727 cells in which we found high EGFR cell surface expression despite rather low total EGFR levels (Fig. 1A and C). It has been reported that EGFR gene amplification and protein expression do not necessarily correlate with each other, and EGFR gene amplification has been associated with improved response to EGFR-targeted therapy, time to progression, and survival (37, 38). In this regard, we
analyzed EGFR gene expression in our panel of NSCLC cell lines by quantitative reverse transcription-PCR and compared it with the EGFR mRNA expression in the A431 cell line, which is known to have multiple copies of the EGFR gene (39). In all NSCLC cell lines, EGFR mRNA levels were low compared with the A431 cell line; however, the Calu6, PC9, and Calu3 cells had moderate expression levels of EGFR mRNA, suggestive of EGFR gene amplification in these cell lines (Fig. 1D). We also compared the levels of constitutive EGFR phosphorylation in the different NSCLC cell lines. The EGFR mutant H1975, H1650, and PC9 cell lines and the EGFR wild-type H322, H441, A549, and H460 cell lines had significantly higher levels of EGFR phosphorylated on tyrosine 1068 (a marker of EGFR activity) than the other cell lines (Fig. 1A and B). In general, there was a close correlation between EGFR protein expression and pEGFR expression (Fig. 1A and B).

Her2 binds no known ligand, but is the preferred dimerization partner for the other erbB family members (40). Furthermore, it has been reported that gefitinib is a potent growth inhibitor of Her2-overexpressing cancer cells (41). Therefore, we determined the basal Her2 and pHer2 expression levels in our panel NSCLC cell lines. The basal levels varied significantly between the different NSCLC cell lines, and there was no correlation with basal pEGFR or pHer2 levels, histologic subtype, or presence of an activating EGFR mutation (Fig. 1A and B). Collectively, these results indicate that there is a close correlation between EGFR expression and activity in this panel of NSCLC cell lines. Furthermore, there seems to be a close correlation between EGFR activity and Her2 activity, suggesting that EGFR/Her2 heterodimers may be active in these cells. Of all the histologic subtypes examined, the bronchoalveolar carcinoma cell lines consistently had the highest pEGFR and pHer2 levels irrespective of EGFR mutational status.

Sensitivity to Single-Agent Gefitinib Does Not Correlate with Histologic Subtype or Constitutive EGFR, Her2, pEGFR, pHer2, pErk1/2, and pAkt Expression

We assessed the antiproliferative activity [IC50(72 hours) values] of gefitinib in the panel of EGFR wild-type and mutant NSCLC cell lines (Fig. 1A and B, top). Most of the EGFR wild-type cell lines were relatively resistant to gefitinib with IC50 values between 7 and 10 μmol/L (Fig. 1A). However, the A549 adenocarcinoma cell line was moderately sensitive (IC50, 4.5 μmol/L), and the H322 bronchoalveolar carcinoma and Calu3 adenocarcinoma cell lines were highly sensitive with IC50 doses of 0.5 and 0.7 μmol/L, respectively (Fig. 1A and B). Among the EGFR mutant NSCLC cell lines, the PC9, a cell line known to have a deletion in exon 19 (delE746A-750) was highly sensitive to gefitinib. However, the A549 and H322 cell lines, which are known to have EGFR mutations, were not as sensitive to gefitinib. The sensitivity of the different cell lines to gefitinib is shown in Figure 2.
gefitinib (IC₅₀ 30 nmol/L). However, the H1650 cell line, which contains the same exon 19 deletion, was resistant to gefitinib (IC₅₀ 9.7 μmol/L). The H1975 cell line, which carries both the L858R (mutation linked with sensitivity to gefitinib) and T790M (mutation linked with resistance to EGFR-TKI) missense mutations (24), was relatively resistant to gefitinib (IC₅₀ 11.7 μmol/L). No correlation was found between sensitivity to gefitinib and constitutive expression or phosphorylation levels of EGFR, Her2, or the downstream signaling molecules Akt and Erk1/2. Nevertheless, among the gefitinib-sensitive cell lines, we found that both the EGFR mutant PC9 and wild-type H322 cell lines contained relatively high basal EGFR and Her2 expression and phosphorylation levels, whereas the Calu3 cell line had relatively high basal Her2 expression and phosphorylation levels. Overall, we found that there was no correlation between sensitivity to gefitinib and EGFR gene mutations, EGFR expression, EGFR phosphorylation, and EGFR gene expression in this panel of NSCLC cell lines.

### Effect of EGF and Gefitinib on Phosphorylation of EGFR, Her2, and Downstream Signaling Molecules

The sensitivity of EGFR mutant NSCLC cell lines to gefitinib was previously correlated with inhibition of pEGFR, pAkt, and pErk1/2 following gefitinib treatment (15, 22). We determined the effect of EGF and gefitinib on EGFR, Her2, Akt, and Erk1/2 phosphorylation in a panel of gefitinib-sensitive and gefitinib-resistant cell lines that were EGFR wild type (Fig. 2A) and mutant (Fig. 2B). In both gefitinib-sensitive and gefitinib-resistant EGFR wild-type cell lines, EGF treatment resulted in activation of pEGFR and pHer2; however, this was not accompanied by an activation of Akt or Erk1/2 (Fig. 2A). Following gefitinib treatment, there was an inhibition of the basal and EGF-stimulated pEGFR and pHer2 phosphorylation in all three cell lines. Notably, pErk1/2 levels were potently down-regulated by gefitinib in the H322 gefitinib-sensitive cell line, but not in the H157 and H460 gefitinib-resistant cell lines (Fig. 2A). pErk1/2 levels were also unaffected by gefitinib in the H23, H727, H838, and H441 gefitinib-resistant EGFR wild-type NSCLC cell lines (data not shown). pAkt levels were also down-regulated in the gefitinib-sensitive H322 cell line, but not as potently as pErk1/2 levels (Fig. 2A). Interestingly, stimulation with EGF resulted in activation of pAkt in all three EGFR mutant cell lines, although only in the gefitinib-sensitive PC9 cell line did this effect seem to be coupled to a simultaneous increase in the levels of pEGFR (Fig. 2B). Gefitinib treatment resulted in inhibition of both pAkt and pErk1/2 in the PC9 gefitinib-sensitive cell line at concentrations as low as 0.01 μmol/L, whereas inhibition of pAkt and pErk1/2 by gefitinib was much less efficient in the H1975 and H1650 cell lines (Fig. 2B). In summary, these data suggest that in the gefitinib-sensitive NSCLC cell lines, the Akt and Erk1/2 pathways are more dependent on signals transduced by EGFR than is the case in the gefitinib-resistant NSCLC cell lines. Furthermore, this is the case irrespective of EGFR mutational status.

### Evaluation of the Antiproliferative Activity of Gefitinib in Combination with Chemotherapy

We next examined the growth inhibitory effects of gefitinib in combination with the chemotherapeutic agent cisplatin. EGFR wild-type and mutant cell lines were exposed to gefitinib and cisplatin in three schedules as described in Materials and Methods (Fig. 3A and B). Significant antagonism was observed between gefitinib and cisplatin in the H838, H727, and H157 EGFR wild-type cells and the H1975 EGFR mutant cell lines with combination indices greater than one in the majority of combinations in all three schedules (Fig. 3A and B; Supplementary Data; data not shown). In the H1650 and PC9 EGFR mutant cell lines, we found an additive/slight antagonistic interaction between cisplatin and gefitinib for all three schedules (Fig. 3B; Supplementary Data). In contrast, the combination induced synergistic growth-inhibitory effects at most concentrations (CI < 1) in the A549, H460, Calu6, and H322 cell lines for all three schedules (Fig. 3A; Supplementary Data; data not shown). Thus, cisplatin interacted synergistically with gefitinib in a subset of EGFR wild-type cell lines, but interestingly interacted antagonistically or at best additively in the EGFR mutant cell lines.

As taxanes, such as Taxol, are frequently used in combination with platinum compounds in the treatment of NSCLC (2), we evaluated the interaction between gefitinib and Taxol. EGFR wild-type and mutant cell lines were exposed to gefitinib and Taxol 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. 4A and B; Supplementary Data). Thus, it seems that EGFR mutational status does not play a role in determining the interaction between gefitinib and Taxol.

### Effect of Gefitinib on Cisplatin- and Taxol-Induced Cell Cycle Arrest and Apoptosis

Flow cytometry was used to examine cell cycle arrest and apoptosis in NSCLC cell lines following treatment with the combination of gefitinib and chemotherapy. In view of our previous data showing similar interactions between gefitinib and the cytotoxic drugs in all three schedules, only the cotreatment schedule was examined. In the H460 cell line, cotreatment with gefitinib and cisplatin resulted in a significant increase in the apoptotic population compared with cells treated with cisplatin alone (23.3% compared with 40.4%, P = 0.0006; Fig. 5, left). In contrast, when H157 were cotreated with cisplatin and gefitinib, a small but significant decrease in apoptosis was observed, compared with cells treated with cisplatin alone (10.5% compared with 5.5%, P = 0.0008; Fig. 5, left). In H838 EGFR wild-type and PC9...
EGFR mutant cells, cotreatment with cisplatin and gefitinib resulted in a decrease in G2-M arrest but no change in the sub-G0-G1 population compared with cisplatin alone. In all cell lines examined, the combination of gefitinib and Taxol resulted in an increase in apoptotic sub-G0-G1 phase (Fig. 5, right). Thus, the results of the cell cycle analysis were consistent with those of the MTT assays.

Effect of Cisplatin and Taxol on EGFR Phosphorylation and Expression

To gain insight into the mechanisms involved in regulating the interaction between chemotherapy and gefitinib, we examined the effect of cisplatin and Taxol on EGFR phosphorylation and expression in a panel of EGFR wild-type and mutant cell lines. In cell lines in which there was a synergistic interaction between cisplatin and gefitinib (the H460, A549, Calu6, and H322 cells), a dose-dependent increase in pEGFR levels was observed 12 to 24 hours after cisplatin exposure, which was accompanied by Akt activation in these cell lines (Fig. 6A; data not shown). This increased EGFR activation was not accompanied by an increase in total and cell surface EGFR expression (Fig. 6A; data not shown). In contrast, in cell lines in which cisplatin and gefitinib were antagonistic (the H157, H838, H727 EGFR wild-type cells and the H1650, H1975, and PC9 EGFR mutant cell lines), cisplatin exposure resulted in no change or a dose-dependent decrease in EGFR and Akt phosphorylation (Fig. 6A, bottom, and B; Table 1; data not shown). Following treatment with Taxol, a dose-dependent increase in EGFR phosphorylation was observed in all EGFR wild-type and mutant cell lines (Fig. 6C and D; Table 1). In all cell lines, this effect was correlated with a dose-dependent activation of Akt (Fig. 6C and D).

Discussion

Somatic “gain-of-function” mutations in EGFR have been identified in NSCLC and many clinical trials suggest that they can be used to predict response to gefitinib and erlotinib. Despite this, several studies have shown that the presence of EGFR mutations was not a perfect predictor of EGFR-TKI effectiveness, as patients without EGFR...
mutations showed response to gefitinib and erlotinib (14–16, 43). Previous studies have suggested that apart from EGFR mutations, other molecular mechanisms can endow the receptor with a “gain-of-function,” leading to tumor cell EGFR dependence and increased sensitivity to EGFR-TKI. In this context, EGFR gene amplification has recently been reported to correlate with response rate to gefitinib (18). In contrast, there are some conflicting data concerning the role of EGFR expression in predicting response to TKIs (28,44). To determine the value of pEGFR as a putative predictive marker, we initially characterized a panel of EGFR wild-type and mutant NSCLC cell lines [H23-H838-A549-Calu3-PC9 (adenocarcinoma cells), H322 bronchoalveolar carcinoma cell lines, and H460 (large cell carcinoma cell line), H727 (carcinoid cell line), and Calu6 (anaplastic carcinoma cell line)] for their response to gefitinib monotherapy. Among the nine EGFR wild-type cells, we found that the H322 bronchoalveolar carcinoma and Calu3 adenocarcinoma cell lines were sensitive to gefitinib monotherapy. There was no clear correlation between histologic subtype and response to gefitinib. In accordance with other studies, no association between

Figure 4. Cell viability assay in NSCLC cell lines in response to gefitinib and Taxol. A and B, crystal violet assays were done in EGFR wild-type H460, A549, H157, and H322 cells and in the EGFR mutant PC9 and H1975 cell lines. Cells were treated with no drug (control), gefitinib alone, Taxol alone, or gefitinib in combination with Taxol for 96 h. The concomitant sequence is presented. To evaluate the interaction between gefitinib and Taxol, we used the method of Chou and Talalay and calculated CI values, where CI < 0.3, 0.3 < CI < 0.7, 0.7 < CI < 0.85, 0.85 < CI < 1, CI = 1, and CI > 1 denotes very strong synergism, strong synergism, moderate synergism, slight synergism, an additive interaction, and antagonism, respectively. Representative results of at least three experiments. Similar results were obtained when cells were either pretreated with gefitinib for 24 h followed by Taxol for 72 h or pretreated with Taxol for 24 h followed by gefitinib for 72 h (Supplementary Data is available online at http://mct.aacrjournals.org).

Figure 5. Determination of cell cycle distribution and apoptosis in cells cotreated with gefitinib and cisplatin or Taxol. Cells were treated concomitantly with gefitinib and cisplatin (left) or Taxol (right) for 72 h and the cell cycle status of the NSCLC cells was monitored by flow cytometry after propidium iodide staining. Representative results of three independent experiments.
sensitivity to gefitinib and constitutive EGFR expression at the protein level, mRNA level, and on the cell surface or pEGFR expression was observed (44). Furthermore, in contrast with some other studies, we did not find any correlation between response to gefitinib and the constitutive Her2 or pHer2 expression (41). Basal activation of Akt or Erk1/2 (42). Surprisingly, the PC9 and H1650 cells, which contain the same in-frame deletion of the EGFR kinase (delE746-A750), showed different responses to gefitinib. In this context, some studies showed that not all patients with EGFR mutations will respond to EGFR-targeted TKI (17, 20). The H1650 did not have any additional mutations, e.g., at codon 790 (T790M); nevertheless, other molecular mechanisms, such as increased internalization of ligand-activated EGFR, the presence of alternative tyrosine kinase receptors, or constitutive activation of downstream signaling pathways, can lead to resistance to EGFR-TKI (26, 45, 46). Collectively, in our panel of NSCLC cell lines, we did not find any additional predictive marker for response to gefitinib monotherapy.

We next evaluated a possible association between sensitivity to gefitinib and inhibition of pEGFR, pHer2, or the downstream signaling molecules pAkt and pErk1/2. Sensitivity to gefitinib did not correlate with inhibition of pEGFR or pHer2. However, in the H322 EGFR wild-type and PC9 EGFR mutant cell lines, which are highly sensitive to gefitinib, there was a strong inhibition of pAkt and pErk1/2 following treatment with gefitinib. In contrast, in the EGFR wild-type and mutant gefitinib-resistant cell lines, there was no inhibition of pErk1/2 and pAkt following treatment with gefitinib. These data strongly suggest that in the gefitinib-resistant NSCLC cell lines, survival pathways, such as the Akt pathway, are not solely dependent on signals transduced by EGFR and Her2.

We also evaluated the effect of combined treatment with gefitinib and chemotherapy. NSCLC cell lines were exposed to gefitinib and chemotherapy in three schedules: concomitant gefitinib and chemotherapy and pretreatment with either gefitinib or chemotherapy, each for 24 hours. Significant antagonism was observed between gefitinib and cisplatin in the H888, H727, and H157 EGFR wild-type cells and in the H1975 EGFR mutant cell lines with combination indices greater than one for all schedules (Table 1). The interaction between gefitinib and cisplatin was additive/slightly antagonistic in the H1650 and PC9 EGFR mutant cell lines. In contrast, the combination of gefitinib and

Figure 6. Effect of cisplatin and Taxol on EGFR and Akt phosphorylation and expression. EGFR wild-type and mutant cells were treated with increasing concentrations of cisplatin (A and B) or Taxol (C and D) for 12 or 24 h, as indicated, and pEGFR, EGFR, pAkt, and Akt levels were determined by Western blotting.
cisplatin induced synergistic growth-inhibitory effects at most concentrations (CI < 1) in the A549, H460, Calu6, and H322 cell lines in all three schedules (Table 1). CI values of 1 (additive interaction) were found in the H23 cell line for all three schedules (Table 1; data not shown). To elucidate the mechanism underlying these effects, we determined drug-induced EGFR expression and phosphorylation following cisplatin exposure. Importantly, the interactions between gefitinib and cisplatin correlated with the effect of cisplatin on EGFR phosphorylation. In the A549, H460, Calu6, and H322 EGFR wild-type cells (cell lines with synergistic interaction between the two agents), a dose-dependent increase in pEGFR was observed following cisplatin treatment and this was accompanied by Akt activation in these cell lines (Table 1). In contrast, in the H727, H838, H157, and H23 EGFR wild-type cell lines and H1650, H1975, and PC9 EGFR mutant cells (cells with antagonistic or additive interaction between the two agents), cisplatin exposure did not result in increased EGFR or Akt phosphorylation (Table 1). We also investigated the interaction between Taxol and gefitinib in these cell lines. In contrast to our data with cisplatin, the interaction between Taxol and gefitinib was synergistic in all EGFR wild-type and mutant cell lines for all three schedules (Table 1). However, in accordance with our cisplatin results, the synergistic interaction between Taxol and gefitinib did correlate with increased EGFR and Akt activation in each cell line following Taxol treatment (Table 1). Collectively, these results suggest that cell lines in which EGFR phosphorylation is increased following drug exposure may depend on EGFR to activate survival pathways (such as Akt). In these cell lines, EGFR might be an “Achilles’ heel,” with chemotherapy-induced activation of EGFR rendering them more sensitive to EGFR inhibition.

The failure of the INTACT-1 and INTACT-2, TALENT, and TRIBUTE trials may have at least partly been due to the lack of selection of patients most likely to benefit from addition of EGFR-targeted TKIs to their chemotherapy regimen. Retrospective analysis for EGFR mutations of 228 tumor specimens of the TRIBUTE trial showed that the response rate to the combination of chemotherapy with erlotinib was greater (53% versus 18%; P = 0.012) in patients with EGFR mutations compared with those with wild-type EGFR (47). However, there was no third treatment arm with erlotinib alone, which was necessary for the complete evaluation of the predictive value of EGFR mutations for the interaction between erlotinib and chemotherapy. Furthermore, retrospective analysis of the INTACT trials showed no statistical difference in overall survival and overall response to combined chemotherapy plus gefitinib treatment in patients with an EGFR mutation or EGF gene amplification (48). Interestingly, in both the H1650 and PC9 EGFR mutant cell lines, we found an additive/slightly antagonistic interaction between cisplatin and gefitinib. This suggests that although the presence of EGFR mutations predicts for sensitivity to TKI in monotherapy, it may not predict response to combined TKI/chemotherapy. However, to be able to generalize our suggestion, confirmation of this observation in a larger number of EGFR mutant cell lines is necessary. Our results suggest that only NSCLC tumors that respond to cisplatin or Taxol by up-regulating EGFR phosphorylation will respond to the addition of EGFR-targeted TKIs to a cisplatin- and/or Taxol-containing regimen. Furthermore, determination of pEGFR levels, before and after drug exposure, as was recently conducted for EGFR levels (49), could be of clinical value in predicting response to combined treatment with cisplatin- and EGFR-targeted TKIs. Moreover, similar results were obtained when chemotherapy (oxaliplatin, 5-fluorouracil, and SN-38) was combined with gefitinib in colorectal cancer cell lines: Only cell lines that responded with increased EGFR phosphorylation following chemotherapy were synergistically growth inhibited following the addition of gefitinib to 5-FU, oxaliplatin, or SN-38 (50). Furthermore, we investigated the mechanism of increased EGFR activation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR status</th>
<th>Effect of cisplatin on pEGFR</th>
<th>Cisplatin-gefitinib interaction</th>
<th>Effect of Taxol on pEGFR</th>
<th>Taxol-gefitinib interaction</th>
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<tbody>
<tr>
<td>A549</td>
<td>WT</td>
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<td>↑</td>
<td>Synergistic</td>
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<tr>
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<td>↑</td>
<td>Synergistic</td>
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<td>↑</td>
<td>Synergistic</td>
</tr>
<tr>
<td>Calu 6</td>
<td>WT</td>
<td>↑</td>
<td>Synergistic</td>
<td>↑</td>
<td>Synergistic</td>
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<tr>
<td>H23</td>
<td>WT</td>
<td>=</td>
<td>Additive</td>
<td>↑</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<tr>
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<td>↓</td>
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<td>Synergistic</td>
</tr>
<tr>
<td>PC9</td>
<td>MT</td>
<td>↓</td>
<td>Antagonistic</td>
<td>↑</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

NOTE: The linespace demarcates the two different phenotypes following treatment with combination of cisplatin and gefitinib. Abbreviations: WT, wild type; MT, mutant; ND, not determined; ↑, increased; =, no change; ↓, decreased.
following chemotherapy in colorectal cancer and NSCLC cells and we found that Src family kinases and metalloproteases mediate chemotherapy-induced EGFR activation in both colorectal cancer and NSCLC cell lines. Moreover, by inhibiting Src family kinases and metalloprotease activity, EGFR-mediated prosurvival signaling in response to chemotherapy was inhibited. 4 Our observation of a synergistic interaction between gefitinib and Taxol in all NSCLC cell lines may indicate that this combination is superior to the cisplatin/gefitinib combination. The ongoing European Organization for Research and Treatment of Cancer 08011 phase III trial, in which the combination of gefitinib and docetaxel was compared with docetaxel with placebo, will indicate if this preclinical observation is clinically relevant.

In summary, by investigating the response of a panel of NSCLC cell lines to gefitinib, we found that both EGFR wild-type and mutant cell lines can be sensitive to gefitinib. Although we were not able to relate gefitinib sensitivity to the constitutive expression or phosphorylation of EGFR, Her2, Akt, or Erk1/2, we have shown that in the gefitinib-sensitive cell lines, inhibition of pEGFR and pHer2 was coupled to inhibition of pAkt and pErk1/2 following gefitinib exposure. Furthermore, modulation of EGFR activity 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 interaction was observed, whereas when drug treatment resulted in decreased EGFR activation, an antagonistic interaction was observed. In addition, the presence of an activating EGFR mutation did not predict response to combined gefitinib/drug treatment. Our findings suggest that measurement of pEGFR expression after drug treatment may identify NSCLC patients most likely to benefit from addition of an EGFR-targeted TKI to their chemotherapy regimen.

4 Data submitted for publication.

Acknowledgments

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References


Molecular Cancer Therapeutics

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Sandra Van Schaeybroeck, Joan Kyula, Donal M. Kelly, et al.


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