Combined Targeting of the Estrogen Receptor and the Epidermal Growth Factor Receptor in Non–Small Cell Lung Cancer Shows Enhanced Antiproliferative Effects

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Abstract

Identifying new effective therapeutic treatments for lung cancer is critical to improving overall patient survival. We have targeted both the estrogen receptor (ER) and the epidermal growth factor receptor (EGFR) pathways using an ER antagonist, fulvestrant ("Faslodex"), and the selective EGFR tyrosine kinase inhibitor, gefitinib ("Iressa"), in non–small cell lung cancer (NSCLC) cells. Rapid activation of phospho-EGFR and phospho-p44/p42 mitogen-activated protein kinase by estrogen was observed, indicating nonnuclear ER transactivation of EGFR. Additionally, EGFR protein expression was down-regulated in response to estrogen and up-regulated in response to fulvestrant in vitro, suggesting that the EGFR pathway is activated when estrogen is depleted in NSCLC cells. Cell growth and apoptosis were examined in several NSCLC lines that express varying amounts of ERβ, EGFR, and Neu but no full-length ERα. One cell line contained an EGFR mutation. Cells were exposed to 10 nmol/L estrogen and 10 ng/mL EGF and either 1 μmol/L fulvestrant or 1 μmol/L gefitinib alone or in combination. In all cell lines, the drug combination decreased cell proliferation up to 90% and increased apoptosis 2-fold. The relative responses to gefitinib and fulvestrant were similar regardless of ER and EGFR expression and mutation status. In an in vitro lung tumor xenograft model, the drug combination decreased tumor volume in severe combined immunodeficient mice by 40% compared with 49% and 32% for gefitinib and fulvestrant treatment alone, respectively. Antitumor effects of the combination therapy were accompanied by biochemical and histologic evidence of increased apoptosis, decreased phospho-p44/p42 mitogen-activated protein kinase expression, and increased Ki-67 expression compared with individual treatment. These studies provide evidence of a functional interaction between the ER and the EGFR pathways in NSCLC.

Introduction

Lung cancer is currently the leading cause of cancer death in U.S. men and women, and surgical resection for cure is often only applicable to early-stage disease. The 5-year survival rate for all stages of lung cancer is only 15% (1). The survival rate is 49% for cases detected when the disease is localized, demonstrating that, even when diagnosed at an early stage, relapse and death from disease are common. Thus, finding new therapeutic treatments for lung cancer is critical. Lung cancer in women has reached epidemic proportions with a 600% increase in death rates from lung cancer in U.S. women from 1930 to 1997.

Several reports of sex differences in lung cancer risk and disease presentation suggest that estrogen may be involved in the etiology of this disease (2). In this respect, we have shown recently that both estrogen receptor (ER) α and β are expressed in non–small cell lung cancer (NSCLC) cell lines, tumor tissues, and cells derived from normal lung (3). Additionally, 17β-estradiol acts as a mitogen for NSCLC cells in vitro and in vivo (3) and can modulate the expression of genes in NSCLC cell lines that are important for control of cell proliferation (4). ERα and ERβ are encoded by separate genes and are members of a large superfamily of nuclear receptors. It is possible that anti-estrogens will have therapeutic value to treat or prevent lung cancer; indeed, we have shown that fulvestrant ("Faslodex"), an ER antagonist with no agonist effects, inhibits lung tumor xenograft growth in severe combined immunodeficient mice by 40% (3).

Fulvestrant is approved in the United States for the management of postmenopausal, ER-positive women with progressive metastatic breast cancer following prior use of anti-estrogen therapy (5). Although this drug is currently not approved for clinical lung cancer treatment, clinical trials assessing its therapeutic value for NSCLC are under way. Fulvestrant exerts its effects by binding to the ER with an affinity similar to that of 17β-estradiol and produces a loss of ER within the tumor. Because lung tumors and normal lung fibroblasts from both males and females express ERs and respond to estrogens, both male and female patients may benefit from anti-estrogen treatment for lung cancer. However, treatment of lung cancer with an anti-estrogenic drug like fulvestrant could give rise to a population of cells with increased levels of epidermal growth factor receptor (EGFR) because of a functional linkage between these two receptors (6). In this regard, nuclear ERs can be activated in the traditional ligand-dependent manner or in a ligand-independent manner that involves EGF-mediated ER phosphorylation. In addition to nuclear ER activation, a nonnuclear ER pool has been proposed in breast cancer to use the EGFR to rapidly signal through various kinase cascades that in turn influence the actions of estrogen.

The EGFR, also known as HER-1 and erbB-1, is part of the tyrosine kinase receptor family that also includes erbB-2 (HER-2/new), erbB-3 (HER-3), and erbB-4 (HER-4; ref. 7) and is important for normal development, differentiation, and cell proliferation (8–10). These four ErbB receptors can either homodimerize or heterodimerize in response to several different ligands, which leads...
to the regulation of transcription factors and other proteins involved in proliferation, cell motility, angiogenesis, cell survival, and differentiation (11, 12). The *EGFR* gene is amplified and/or overexpressed in many human cancers, including both cell lines and tissue specimens of NSCLC, and this often correlates with more aggressive disease, higher incidence of lymph node metastasis, and poor prognosis (13, 14).

Gefitinib (“Iressa”), an EGFR receptor tyrosine kinase inhibitor, is approved for clinical use in the treatment of advanced NSCLC as monotherapy following failure of chemotherapy (15). Response rates to gefitinib in two phase II trials (IDEAL 1 and 2) ranged from 11.8% to 18.4% (16, 17). The patients in the IDEAL trials had received at least one prior chemotherapy regimen, and women in these trials experienced a higher response rate than men (16).

Additionally, the presence of adenocarcinoma and being a never smoker have also been shown to be independent predictors of response (18), with both demographic factors often composed mainly of women. Furthermore, a case report was described of a 52-year-old woman with stage IV NSCLC who achieved a rapid, dramatic clinical response to gefitinib (19) as well as two additional case reports of rapid improvement in women with bronchoalveolar carcinoma (20). These observations suggest that interactions between the EGFR signal transduction pathway and the estrogen stimulatory pathway may be important in the activity of gefitinib in female lung cancer patients. Recently, activating mutations in the tyrosine kinase domain of the EGFR have been identified which may be responsible for the clinical responsiveness to gefitinib (21, 22). These mutations occur more frequently in tumors from female patients, which suggests that cross-activation of EGFR signaling by the ER might promote the development of tumors containing EGFR mutations.

Gefitinib has also been tested in combination with chemotherapy in the large clinical INTACT 1 and 2 phase III trials (23, 24). However, the combination therapies did not produce any additional therapeutic benefit over chemotherapy treatment alone, suggesting that the concurrent use of gefitinib with cytotoxics negates the effects of gefitinib. A combination of an anti-estrogen with gefitinib, both of which could suppress the G1-S cell cycle transition by different mechanisms, could be beneficial. Recent results using breast cancer cells support the use of this combination (25).

We hypothesize that by blocking both the ER and the EGFR pathways we can enhance antitumor effects above that seen by either agent alone. The goals of this study were to show the interaction between the ER and the EGFR pathways in lung cancer cells and to target these two pathways with combination drug therapy. In the present study, we have found that EGFR protein expression is down-regulated in response to estrogen and up-regulated in response to anti-estrogens in vitro. Conversely, ERβ expression is decreased in response to EGF and increased in response to gefitinib. This suggests that the EGFR pathway is more activated when estrogen is depleted in lung cancer cells, establishing a rationale to use this combined therapy. Additionally, we have shown both *in vitro* and *in vivo* that the combination of fulvestrant and gefitinib in NSCLC can maximally inhibit cell proliferation, induce apoptosis, and affect downstream signaling pathways. The cross-signaling between the EGFR/ER pathways in the lung, the striking efficacy of gefitinib in female lung cancer patients, and the emerging role of estrogens in lung tumorigenesis provide rationale to combine gefitinib with anti-estrogen therapy for lung cancer treatment.

### Materials and Methods

**Reagents.** Gefitinib was a kind gift from AstraZeneca (Macclesfield, United Kingdom). Fulvestrant was purchased from Tocris (Ellisville, MO). 17β-Estradiol (1.7 mg, 60-day release) and placebo pellets were from Innovative Research of America (Sarasota, FL). Human recombinant EGF, anti–heparin binding EGF (HB-EGF), anti–transforming growth factor-α (TGF-α), TG-F-α ELISA kit, and amphiregulin ELISA kit was obtained from Oncogene Research Products (San Diego, CA). Anti-amphiregulin was obtained from R&D Systems (Minneapolis, MN). The EGFR blocking antibody M225 was obtained from Imclone Systems, Inc. (New York, NY). Marimistat was obtained from British Biotech (Oxford, United Kingdom). CRM197 was purchased from List Biological Laboratories (Campbell, CA). All PCR reagents were obtained from Applied Biosystems (Foster City, CA).

**Cell Lines and Culture Conditions.** NSCLC cell lines 201T and 273T were established in our laboratory from primary tissue specimens (26). These lung tumors were verified to be primaries to the lung by pathologic assessment. These cells were maintained in *vitro* in basal medium Eagle (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics. A549 cells (bronchioalveolar carcinoma) and MCF7 cells (breast adenocarcinoma) were purchased from the American Type Culture Collection (Rockville, MD) and maintained in basal medium Eagle supplemented with 1% or 10% fetal bovine serum, respectively.

**Protein Extraction and Western Analysis.** Cells were grown in T75 flasks and experimental treatments were added as indicated in the figure legends. For cells treated with estrogen or EGF, cells were serum deprived for 48 to 72 hours before treatment. Following experimental treatment, cells were washed once with ice-cold PBS. Protein was extracted by adding 300 μL ice-cold radioimmunoprecipitation assay buffer [1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL phenylmethylsulfonyl fluoride, 0.045 mg/mL aprotinin (Sigma Chemical), 1 mmol/L sodium orthovanadate] per flask and scraped into a microfuge tube. The cell lysate was passed through a 21-gauge needle to shear genomic DNA. Phenylmethylsulfonyl fluoride stock (10 μL, 10 mg/mL) was added and incubated for 30 to 60 minutes on ice. The cell lysate was microcentrifuged at 10,000 × g for 10 minutes at 4°C. Protein concentration in the supernatant was measured using the BCA-200 Protein Assay kit (Pierce, Rockford, IL).

For detection of proteins, equal amounts of cell lysate (50-100 μg) were separated by size on a 10% SDS-Tricine gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Nonspecific binding sites were blocked by incubation in 1× TBST (0.2 mol/L Tris, 0.14 mol/L NaCl, 0.1% Tween 20) containing 5% dry milk for 2 hours at room temperature followed by incubation overnight at 4°C with primary antibody. The following antibodies and dilutions were used: anti-EGFR monoclonal antibody (Transduction Laboratories, Lexington, KY), 1:1,000 dilution; anti– Neu polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1,000 dilution; phosho-p44/p42 mitogen-activated protein kinase (MAPK) monoclonal antibody (Cell Signaling Technology, Beverly, MA), 1:1,000 dilution; anti–ERα polyclonal antibody (HC-20, Santa Cruz Biotechnology), 1:1,000 dilution; and anti–ERβ polyclonal antibody (PanVera, Madison, WI), 1:1,000 dilution. All antibodies were added in 1× TBST containing 1% dry milk. After primary antibody incubation, the blots were washed thrice in 1× TBST (10 minutes each at room temperature), and horseradish peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG (Amersham, Piscataway, NJ) was added at a 1:2,000 dilution for all polyclonal antibodies or monoclonal antibodies, respectively. All secondary antibodies were incubated for 2 hours at room temperature. After three more washes with 1× TBST, the immunoreactive peptide was detected by SuperSignal West Pico Chemiluminescent Detection kit (Pierce, Rockford, IL).

### References

For tumor xenografts, tumors were removed after the 4-week treatment period and a section for protein extraction was frozen in liquid nitrogen. Tumor tissue was weighed and diced into small pieces using a clean razor blade. Protein was extracted by adding 3 mL ice-cold radioimmunoprecipitation assay buffer per gram of tumor and homogenized using a polytron. Phenylmethylsulfonyl fluoride stock (20 µL, 10 mg/mL) were added to each lysate and incubated on ice for 30 to 60 minutes. The cell lysate was centrifuged at 10,000 x g for 10 minutes at 4°C. The protein concentration in the supernatant was measured using the BCA-200 Protein Assay kit.

Isolation of Genomic DNA and EGFR Mutation Analysis. Genomic DNA was isolated from each cell line using the PUREGENE DNA Purification kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol. EGFR exons 18 to 21 and flanking intron DNA were amplified by PCR using the same primers and conditions described by Paez et al. (22). Each PCR reaction contained 20 ng DNA, 1 x PCR buffer, 0.125 mM forward and reverse primers in a 20 µL reaction volume. PCR products were resolved on an agarose gel and DNA was purified using the QiAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Both forward and reverse sequencing fragments were analyzed via capillary electrophoresis using ABI prism 3700 DNA analyzer (Applied Biosystems) and Vector NTI followed by manual review. Candidate mutation samples in both directions were reamplified and resequenced as described above.

ELISA Assay. Cells (2 x 10^6 cells/mL) were plated in 10 cm² plates and allowed to attach overnight. The cells were washed with PBS and incubated in serum-free phenol red-free medium for 72 hours. Cells were treated with 10 mM estrogen for 10 minutes. Supernatants were collected and cells were centrifuged at 1,200 x g for 10 minutes. The resulting supernatants were each concentrated to 300 µL using an Amicon ultrafilter device and tested in duplicate for levels of TGF-α, HB-EGF, and amphiregulin by ELISA following the manufacturer's instructions.

Cell Proliferation Assay. Cells were plated on 96-well plates at a density of 3.5 x 10^3 cells/well and allowed to attach overnight. The cells were washed with PBS and incubated in serum-free phenol red-free medium for 48 hours. Treatments were added as indicated in the figure legends for 72 hours. Samples were analyzed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following the manufacturer's instructions. Absorbance values at 490 nm were recorded 1 hour following addition of the CellTiter 96 One Solution Reagent (provided in kit) using a Wallac Victor2 1420 Multilabel Counter, and readings were directly proportional to the amount of cellular proliferation.

Apoptosis Assay. Cells were plated on 96-well plates at a density of 3.5 x 10^3 cells/well and allowed to attach overnight. The cells were washed with PBS and incubated in serum-free phenol red-free medium for 48 hours. Treatments were added as indicated in the figure legends and incubated for 72 hours. Samples were analyzed using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) following the manufacturer's instructions. Fluorescence was measured in each well using a Wallac Victor2 1420 Multilabel Counter and these values were directly proportional to amount of apoptotic induction.

For analysis of lung tumor xenograft cell extracts, 25 µg of each cell extract were added in duplicate directly to the wells of a 96-well plate in a 100 µL volume. Samples were immediately analyzed as above.

In vivo Tumor Xenograft Model. 17β-Estradiol pellets (1.7 mg, 60-day release) or placebo pellets were implanted s.c. into 40 female severe combined immunodeficient mice (20 estrogen-treated and 20 placebo-treated controls, 5 weeks old, Harlan Sprague-Dawley, Indianapolis, IN). Three days after pellet implantation, 201T lung tumor cells were injected s.c. at two sites per mouse (2 x 10^5 cells per site). The mice were divided into eight treatment groups (five animals per group): (a) placebo, (b) estrogen, (c) estrogen plus fulvestrant, (d) estrogen plus gefitinib, (e) estrogen plus gefitinib plus gefitinib, (f) placebo plus fulvestrant, (g) placebo plus gefitinib, and (h) placebo plus fulvestrant plus gefitinib.

Fulvestrant (30 mg/kg of mouse weight) or vehicle control (peanut oil) was injected s.c. twice a week for 4 weeks. Treatment started 2 days following pellet implantation. Gefitinib was given at a dose of 150 mg/kg mouse daily by oral gavage for 4 weeks. Tumor size was measured each week and reported as an average relative tumor volume calculated as (l x w x h) / 2 (mm³), where l is the length, w is the width, and h is the height of the tumor measured with calipers. At the end of the 4-week period, the animals were sacrificed and the tumors were removed. Half of the tumor was harvested for protein analysis and the other half was fixed in 10% formalin for immunohistochemical analysis. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

Immunohistochemical Staining. Lung tumor xenograft samples were fixed in 10% formalin for 15 to 30 minutes at room temperature and stored in 100% ethanol. Tissues were then paraffin embedded, sliced, and mounted on slides. Paraffin was removed from the slides with xylenes and the slides were stained according to standard procedures. Primary antibody was anti-phospho-p44/p42 MAPK or anti-Ki-67 (DakoCytomation, Carpinteria, CA) at a 1:100 dilution. The secondary antibody was a biotinylated IgG specific for the primary antibody. Brown staining was considered positive. Negative control staining was done without the addition of primary. For Ki-67 quantitation, three slides per experimental treatment were read and scored for the number of positive cells per five high-powered fields. For quantitation of number of tumor nuclei in H&E-stained slides, two slides per experimental treatment were read and scored for the number of positive cells per five high-powered fields. Results are reported as the mean ± SE. Statistical analysis was done using an unpaired t test.

Results

Expression of EGFR and ER in Lung Cancer Cell Lines. We first examined protein expression of the EGF and ERs in the cell lines used for these studies. Each cell line represents a different histologic type of tumor: 201T, adenocarcinoma; A549, bronchoalveolar carcinoma; and 273T, squamous cell carcinoma. 273T cells were derived from a female patient, whereas both 201T and A549 were derived from male patients. All three lung cancer cell lines expressed EGFR and ERβ albeit to different extents (Fig. 1; Table 1). The 273T squamous carcinoma cell line overexpressed both receptors. A549 and MCF7 cells showed an inverse correlation between EGFR and ERβ

Figure 1. Immunoblots of whole cell lysates from lung tumor cell lines 201T, A549, and 273T and MCF7 breast cancer cells. Cell extracts were prepared from lung tumor cell lines and one breast cancer cell line and 50 µg of each sample were analyzed by Western blotting using a mouse monoclonal anti-EGFR antibody. Blots were stripped and reprobed using a rabbit polyclonal anti-Neu antibody, a rabbit polyclonal anti-ERα antibody, a rabbit polyclonal anti-ERβ antibody, and a mouse monoclonal β-actin antibody.
expression, a correlation that has been reported previously for breast cancer cells (27) and primary breast cancer tissues (28). EGFR expression was observed in the immunoblot of MCF7 breast cancer cells (27) and primary breast cancer tissues (28).

We have shown previously that ERα is composed mainly of alternatively spliced variants and found mostly in nonnuclear compartments and that ERβ is probably the predominant functional nuclear receptor (3). Some lung cancer cell lines do express full-length ERα with relatively low levels of expression. Furthermore, because estrogen activates transcription when complexed to ERα and inhibits transcription with ERβ from AP-1 sites (29), we transiently transfected an AP-1 luciferase construct into lung tumor cells. We observed a slight decrease in transcriptional activation from AP-1 sites (29), we transiently transfected an AP-1 luciferase construct into lung tumor cells. We observed a slight decrease in transcriptional activation with EGF and estrogen, respectively. To determine if the ERs in lung cancer cells can transactivate the EGFR and activate downstream signaling pathways, the cells were treated for 5 minutes with 10 ng/mL EGF, 10 nmol/L estradiol, or a combination of the two treatments (Fig. 2). A 2.6- and 2.8-fold stimulation of phospho-p44/p42 MAPK was observed with EGF and estrogen, respectively.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>EGFR gene mutation</th>
<th>Amino acid substitution</th>
<th>Relative EGFR protein expression</th>
<th>Relative Neu protein expression</th>
<th>Relative ERβ protein expression</th>
<th>IC50 for gefitinib (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>201T</td>
<td>Adenocarcinoma</td>
<td>None</td>
<td>None</td>
<td>32</td>
<td>35</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>A549</td>
<td>Bronchoalveolar carcinoma</td>
<td>None</td>
<td>None</td>
<td>42</td>
<td>51</td>
<td>9</td>
<td>40</td>
</tr>
<tr>
<td>273T</td>
<td>Squamous</td>
<td>A to G (nucleotide 2,180)</td>
<td>Y727C</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>17</td>
</tr>
</tbody>
</table>

NOTE: The EGFR tyrosine kinase domain was sequenced in 201T, A549, and 273T cell lines. A mutation of the EGFR gene was found in 273T cells located at nucleotide 2,180 within exon 18 resulting in a change of Tyr727 to Cys. EGFR, Neu, and ERβ protein expression was quantitated using densitometry and ImageQuant analysis and expressed relative to the intensity of the signal in 273T cells. The IC50 for gefitinib was calculated under normal growth conditions using a MTS assay.

Figure 2. Stimulation of phospho-p44/p42 MAPK by EGF and estrogen. 201T lung cancer cells were serum deprived for 48 hours followed by treatment with 10 ng/mL EGF, 10 nmol/L estradiol (β-E2), or a combination for 5 minutes. Cell extracts were prepared and 30 µg of each sample were analyzed by Western blotting using an anti-phospho-p44/p42 MAPK antibody. The blot was stripped and reprobed with an anti-p44/p42 MAPK antibody (total). Quantitation was done using densitometry and ImageQuant analysis. No treatment (none) was set to 100 and fold increases were calculated over no treatment. Average results of two samples per treatment of a representative experiment that was repeated two additional times.

Another member of the EGFR family, Neu, was also examined in these cell lines and was present in all cell lines examined to different extents (Fig. 1; Table 1). There are currently no known ligands for Neu, but this receptor can form heterodimers with other HER family members (30). β-actin protein expression showed no difference between these cell lines. Reproducibility of protein expression levels was confirmed in at least two separate experiments.

We also examined EGFR mutation status in the cell lines examined in this study (Table 1), because recent evidence suggests that activating mutations in the tyrosine kinase domain of the EGFR may be responsible for the clinical responsiveness to gefitinib (21, 22). There are no mutations found in exons 18 to 21 of the EGFR gene in the 201T and A549 cell lines. A heterozygous point mutation was detected in 273T cells within exon 18 at nucleotide position 2,180. The nucleotide substitution is from A to G, and results in the substitution of amino acid from tyrosine to cysteine at codon 727. This mutation lies in the glycine-rich P-loop of the tyrosine kinase domain. To our knowledge, this is a novel mutation that has not been identified in any other screening studies. The 273T cell line also the highest EGFR protein expression level compared with the 201T and A549 cell lines that do not contain a mutation in this region. This suggests that there is a correlation between EGFR activating mutations and EGFR overexpression possibly due to repositioning of critical residues, which activate transcription. ERβ and Neu expression was also the highest in the 273T cell line. Despite the EGFR mutation, the response of 273T cells to gefitinib was very similar to 201T and A549. In this regard, the IC50 values for gefitinib under normal growth conditions for each of the cell lines that we analyzed are 40, 20, and 17 µmol/L for A549, 201T, and 273T, respectively.

Rapid Stimulation of Phospho-p44/p42 MAPK by Estrogen and EGF. To determine if the ERs in lung cancer cells can transactivate the EGFR and activate downstream signaling pathways, the cells were treated for 5 minutes with 10 ng/mL EGF, 10 nmol/L 17β-estradiol, or a combination of the two treatments (Fig. 2). A 2.6- and 2.8-fold stimulation of phospho-p44/p42 MAPK was observed with EGF and estrogen, respectively.
The combination achieved a more than additive phospho-p44/p42 MAPK stimulation of 13.5-fold ($P < 0.0005$ compared with either fulvestrant or gefitinib single-agent treatment). These rapid responses are indicative of a transactivation of EGFR by ER. Phospho-EGFR was also activated by estrogen treatment within 5 minutes (data not shown). These results have been observed in two additional independent experiments.

The estrogen-induced phospho-p44/p42 MAPK induction is dependent on the EGFR. Figure 3A and B show that the EGFR blocking antibody, M225, can almost completely abrogate (>80% inhibition) the 2-fold phospho-p44/p42 MAPK induction by estrogen. Additionally, matrix metalloproteinases are required for this activation, as the matrix metalloproteinase inhibitor, marimastat, can also completely inhibit this induction by >80%. Several ligands seem to be involved in this response. For example, pretreatment of cells with inhibitors of HB-EGF, CRM197 (a mutated form of diphtheria toxin) and a HB-EGF neutralizing antibody, showed significant changes in estrogen-induced phospho-p44/p42 MAPK expression (Fig. 3C). Pretreatment with neutralizing antibodies for TGF-α and amphiregulin also showed significant changes in estrogen-induced phospho-p44/p42 MAPK expression. Additionally, ELISA assays for each of these three ligands revealed 4.4-, 1.2-, and 1.8-fold releases of TGF-α, amphiregulin, and HB-EGF, respectively, in the supernatant on estrogen stimulation (Fig. 3D). These results suggest that TGF-α and HB-EGF are the main ligands involved in this response and support the functional interaction between ER and EGFR in lung cancer cells.

**Estrogen Modulates EGFR Levels and EGF Modulates ER Levels.** We chose to examine the ER antagonist, fulvestrant, as a target for ER signaling disruption, because tamoxifen (a widely

![Figure 3. Estrogen-induced phospho-p44/p42 MAPK depends on EGFR activation.](image-url)
studied anti-estrogen) increased estrogen-induced in vivo lung tumor xenograft growth in mice by ~1.5-fold, whereas fulvestrant inhibited growth by >40% over estrogen-treated controls (ref. 3; data not shown). This suggests that tamoxifen has partial agonist activity in the lung, similar to that observed in the endometrium. In cells that are cultured for 7 days in the presence of either 10 nmol/L 17β-estradiol or 1 µmol/L fulvestrant, we observed that EGFR levels were modulated by estrogen (Fig. 4A). For example, EGFR expression was decreased by 33% in cells treated with charcoal-stripped serum and 17β-estradiol simultaneously. This suggests that the presence of estrogen alone is sufficient to decrease EGFR expression. Conversely, treatment of lung cancer cells with fulvestrant was able to induce EGFR expression by 2.7-fold. Although EGFR is an estrogen-induced gene, its expression is suppressed with 7-day estrogen treatment. This has been observed previously in breast cancer cells (31). This suggests that growth-inducing events activated by estrogen also act to suppress the EGFR.

On the other hand, when cells are treated with either EGF or gefitinib for 7 days, ERβ can be down-regulated by 41% in response to EGF treatment, whereas gefitinib can increase ERβ expression by 5.4-fold compared with cells without any treatment (Fig. 4B). This reciprocal control mechanism provides strong rationale for combination therapy using these two drugs. These experiments have been repeated in two additional cell lines with similar results (data not shown).

**Cellular Proliferation is Decreased and Apoptosis is Increased in Response to Fulvestrant and Gefitinib Treatment In vitro.** We next examined cell growth in vitro using a standard MTS assay. Briefly, lung cancer cells were exposed to 10 nmol/L estrogen and 10 ng/mL EGF and either 1 µmol/L fulvestrant, 1 µmol/L gefitinib, or a combination of these drugs (Fig. 5A). In all three of these cell lines examined, EGF and estrogen induced up to a 2-fold increase in cell proliferation. In A549 cells, fulvestrant and gefitinib treatment alone inhibited the EGF and estrogen–induced growth by 85% and 83%, respectively. In 201T cells, fulvestrant inhibited this stimulation by 34% and gefitinib inhibited cell growth by 55%. In 273T cells, fulvestrant alone inhibited the estrogen and EGF induction by 45%, whereas gefitinib alone inhibited growth by 68%. Gefitinib treatment alone inhibited cell growth more than fulvestrant alone in the 201T and 273T cell lines. In A549 cells, the addition of either drug alone inhibited cellular proliferation by >80%; the reason for the difference in the A549 cell line is not known at the present time. A dramatic decrease in cell proliferation (90% decreases in A549 and 201T cells and 78% decrease in 273T cells) was observed in estrogen and EGF–treated cells when exposed to a combination of fulvestrant and gefitinib. Although these decreases were more than with either treatment alone, synergism with these two drugs was not observed.

Two of the three cell lines that were analyzed in this study show statistical significance between the gefitinib and the fulvestrant plus gefitinib treatments. Statistical analysis shows that in the cell proliferation assays using 201T cells (Fig. 5A) there was a statistically significant difference between fulvestrant and gefitinib alone and fulvestrant plus gefitinib together in stimulated cells ($P = 0.0001$ and 0.0008, respectively). In the 273T cell line, the $P$ for fulvestrant versus fulvestrant plus gefitinib was not quite significant ($P = 0.14$) and the $P$ for gefitinib versus fulvestrant plus gefitinib in stimulated cells was 0.0019. The A549 cell line was the only cell line that did not show statistical differences when analyzing for dual blockade versus single treatment. At the concentrations used (1 µmol/L for each drug), single treatment alone achieved >80% inhibition of A549 cell growth, whereas the dual treatment inhibited estrogen and EGF stimulated growth by 90%. The A549 cell line does not contain an EGFR mutation in the tyrosine kinase domain, which renders sensitivity to gefitinib.

In unstimulated cells (data not shown), either gefitinib and fulvestrant alone versus combined gefitinib and fulvestrant also achieved statistically significant differences in all cell lines examined. For example, in 201T cells, $P = 0.0001$ for fulvestrant versus fulvestrant plus gefitinib and $P = 0.003$ for gefitinib versus fulvestrant plus gefitinib. These results suggest that dual blockade is indeed superior over single-agent blockade in vitro. The main difference in the unstimulated cells is that the fulvestrant response is not as great as in cells that have been treated with exogenous estrogen. For example, in 201T cells, we observed only a 10% decrease in cell proliferation with fulvestrant alone, 24% with gefitinib alone, and 78% with the combination therapy. These results suggest that even in the absence of added ligand these drugs can exert their effects.

The striking changes in estrogen and EGF–induced cell proliferation in lung cancer cell lines due to the combined fulvestrant and gefitinib treatment were accompanied by an ~2-fold increase in apoptosis (1.3-, 2.0-, and 2.1-fold increase in A549, 201T, and 273T, respectively; Fig. 5B). As with the cell growth assays, these changes with the combination treatment were greater than the changes observed with either drug alone. For example, in A549 cells, fulvestrant treated cells showed no significant change in apoptosis over the EGF and estrogen–treated cells and gefitinib–treated cells increased apoptosis by 1.1-fold. In 201T cells, fulvestrant alone stimulated apoptosis by 1.3-fold, whereas gefitinib increased apoptotic levels by 1.5-fold. The same trend was observed with 273T cells; fulvestrant and gefitinib alone stimulated apoptosis...
by 1.1- and 1.2-fold, respectively. This assay measures the activities of caspase-3 and caspase-7, which have key roles in apoptosis. There were statistically significant differences in single-agent versus dual-agent treatment in the apoptosis assay using the 201T cell line (Fig. 5B; \( P = 0.0045 \) for fulvestrant versus fulvestrant plus gefitinib and \( P = 0.012 \) for gefitinib versus fulvestrant plus gefitinib in stimulated cells). Statistical data for the apoptosis assay in the 273T cell line were \( P = 0.02 \) for both comparisons, whereas the data for these comparisons in A549 cells were not quite significant (\( P = 0.10 \) for both comparisons). Similar results were obtained in cells that were not stimulated with estrogen and EGF (data not shown). Together, these results suggest that combination therapy can provide maximum therapeutic benefits over either treatment alone and these results were statistically significant.

To optimally design the in vivo animal experiment to test these drugs, we have also examined cell proliferation using different drug...
controls (and presence of exogenous estrogen, respectively, compared with line was also used and showed similar results. The combination repeated two additional times with similar results. The 273T cell compared with placebo-treated controls. This experiment was combination treatment inhibited growth by 68.4% (P < 0.001)

In vivo Tumor Growth in Immunocompromised Mice. Growth experiments were conducted in vivo to assess whether the combination of fulvestrant and gefitinib could maximally inhibit growth in immunocompromised mice over either treatment alone. The 201T cell line that was used in this experiment showed expression of both ERβ1 and EGFR and was highly responsive to these drugs in vitro (Figs. 1 and 5). Figure 6 shows a representative experiment with five animals per treatment group. As shown in Fig. 6, estrogen significantly stimulated tumor growth by 2.4-fold compared with placebo-treated controls (P < 0.001). This is consistent with our previous reports using the H23 lung cell line (3). In the presence of exogenous estrogen, fulvestrant and gefitinib alone significantly inhibited tumor growth by 32.2% and 49.7%, respectively (P < 0.05 for fulvestrant versus estrogen and P < 0.001 for gefitinib versus estrogen). The combination had a greater effect with a 59.4% decrease in tumor growth (P < 0.001). The same effects are observed in the placebo-treated controls. In this respect, fulvestrant and gefitinib inhibited tumor growth by 33.7% and 61%, respectively (P < 0.05 and P < 0.01) and the combination treatment inhibited growth by 68.4% (P < 0.001) compared with placebo-treated controls. This experiment was repeated two additional times with similar results. The 273T cell line was also used and showed similar results. The combination of fulvestrant and gefitinib treatment in this line achieved an 82.7% and 76.5% decrease in tumor volume in the absence and presence of exogenous estrogen, respectively, compared with controls (P < 0.0001; data not shown).

Although the tumor volumes were smaller with combination treatment compared with individual drug treatment and the fulvestrant tumor volume versus fulvestrant plus gefitinib tumor volume was statistically different, the gefitinib tumor volume versus fulvestrant plus gefitinib did not show statistically significant differences in the presence or absence of exogenous estrogen. This could be due to a less than optimal dosing regimen or a need for a larger number of animals per group. Nevertheless, there was strong histologic evidence that the combination therapy was more effective based on tumor cellularity and status of signaling molecules. Lung tumor xenografts were removed and histologic changes between the treatment groups were examined (Fig. 7). Distinct glandular structures are observed along with prominent stromal components in the tumors from animals treated with fulvestrant and gefitinib (Fig. 7B) versus controls (Fig. 7A). The tumors from animals treated with combination therapy have a much lower tumor cell content versus the tumors from control treated animals, which seem highly unorganized with densely packed malignant cells and little stroma. Additionally, on a higher magnification, the tumors from animals treated with combination therapy have very dark and dense nuclei and smaller nucleoli versus the tumors from control treated animals, indicative of degenerative cells undergoing apoptosis. These cytologic changes were most pronounced in the combination treatment group and were observed in every tumor from this group. Less pronounced histologic changes were observed in the fulvestrant and gefitinib alone–treated groups but not to the extent as in the combination–treated tumors (not pictured). Quantitation of the number of tumor nuclei present in tumors from control–treated animals versus combination–treated animals was decreased by 62% and this was statistically significant (mean ± SE number of tumor nuclei per field, 312.4 ± 14.1 versus 112.0 ± 5.2, respectively; P < 0.0001). Tumors from animals treated with fulvestrant or gefitinib alone had 235.4 ± 114.1 or 145.2 ± 12.1 mean number of tumor nuclei per field, respectively, both of which are statistically higher than the results from the combination–treated group (P < 0.0001 and P = 0.0245, respectively).

In vivo Signaling Is Affected by Combination Fulvestrant and Gefitinib Treatment. Frozen tumor sections from the in vivo lung tumor xenograft experiment were harvested for protein and used in an apoptosis assay. The amount of apoptotic induction by fulvestrant and gefitinib was 2-fold over the control treatment.
sections from tumors from control treated animals, whereas Fig. 9B and C represent sections from tumors from fulvestrant alone or gefitinib alone–treated animals after the 4-week treatment period. The phospho-p44/p42 MAPK expression is observed uniformly throughout the control-treated tumors. The treatments shown in Fig. 4B to D are representative of the areas of the slide with the maximum amount of staining. The expression of phospho-p44/p42 MAPK in the combination treatment group (Fig. 9D) is almost completely abrogated compared with expression in the control group or the single-agent group.

Discussion

We have published previously a report showing that NSCLC cell lines and in vivo tumor xenografts respond to estrogens and tumor growth can be inhibited by 40% by fulvestrant (3). In the present study, we have targeted both the ER and the EGFR in lung cancer cells with fulvestrant and gefitinib, respectively, to achieve greater antiproliferative effects over either agent alone. We have presented several lines of evidence, which support a functional interaction between these two pathways in the lung. First, phospho-EGFR and phospho-p44/p42 MAPK can be rapidly activated by estrogen and EGF. Second, EGFR ligands are released on estrogen stimulation in vitro. Third, estrogen and was statistically significant (Fig. 8A). Fulvestrant and gefitinib treatment alone each gave 1.6-fold increases over control. These results were confirmed by an additional method using the ApoTag in situ hybridization kit (Intergen, Purchase, NY; data not shown).

Additionally, the amount of cellular proliferation, as analyzed by Ki-67 immunostaining, was markedly decreased in the tumors treated with fulvestrant and gefitinib (Fig. 8B). Relative Ki-67 protein expression was decreased by 35.9% and 51.4% in tumors treated with fulvestrant or gefitinib alone, respectively, versus control treated tumors. Ki-67 expression was decreased by 68.2% in tumors treated with both fulvestrant and gefitinib. The inhibition of Ki-67 by dual receptor blockade was statistically different from that observed with either fulvestrant or gefitinib treatment alone (P = 0.043 and 0.0028, respectively).

We next examined expression of phospho-p44/p42 MAPK in paraffin sections of the tumors removed from the animals. Figure 9A shows phospho-p44/p42 MAPK immunostaining of
can modulate EGFR levels, and conversely, EGF can modulate ER levels in vitro. Finally, tumor proliferation and apoptosis can be maximally inhibited in vitro and downstream signaling molecules, including phospho-p44/p42 MAPK, can be significantly affected in vivo by the combination of fulvestrant and gefitinib.

Hormonal therapy for lung cancer treatment is a novel approach that is currently being explored based on the observations that ERs are indeed expressed in lung cells and show biological responses to estrogens. In particular, ER$^\beta$ seems to play a predominant role in lung cancer (3). Estrogens have been shown to act via ER$^\beta$ in the lung to directly regulate platelet-derived growth factor A and granulocyte-macrophage colony-stimulating factor, key regulators of alveolar formation and surfactant homeostasis, respectively (32). Furthermore, Omoto et al. has shown that ER$^\beta$ expression is stronger in cancerous samples than normal samples (33). A better understanding of the role of ER$^\beta$ in the lung is necessary to lead to novel strategies and targets for lung cancer treatment and prevention.

There are currently three available strategies to target the estrogen signaling pathway in cancer cells. These include the following approaches: (a) antagonists of ER function through drugs, such as tamoxifen and raloxifene; (b) down-regulation of ER function through agents, such as those used in this particular study (fulvestrant); and (c) reduction of estrogen levels through aromatase inhibitors, such as the reversible nonsteroidal agents letrozole and anastrozole (34) and the irreversible steroidal inactivator exemestane (35). Tamoxifen and raloxifene have partial agonistic effects in tissues, such as endometrium. Our laboratory has shown that tamoxifen increases lung tumor xenograft growth and is not an appropriate choice of therapy for NSCLC. Additionally, results from the Tamoxifen Breast Cancer Prevention Trial as part of the National Surgical Adjuvant Breast and Bowel Project did not show any decreased risk of lung cancer (36). Seventeen tumors of the lung, trachea, and bronchus were reported among the placebo group and 20 in the women who had received tamoxifen therapy. Although not statistically significant, these results do suggest that tamoxifen may have some agonistic...
effects in the lung, supporting our observations. The aromatase enzyme catalyzes the conversion of androgens to estrogens and is expressed in the lung (37, 38). No preclinical work has been done using aromatase inhibitors for lung cancer therapy; however, a recent report suggests that this approach may be worth pursuing (39). In this respect, Coombes et al. reported a decreased incidence of primary lung cancer in breast cancer patients treated with exemestane after 2 to 3 years of tamoxifen therapy (4 cases) compared with continued tamoxifen treatment (12 cases). Because lung tumors from both male and female patients express ERs and cell lines derived from both sexes respond to estrogens and anti-estrogens, these types of therapeutic treatments could be useful for both populations, not just women (3).

There are also several strategies that have been developed to target EGFR and block its activation in cancer cells. Inhibitors of this pathway include monoclonal antibodies, such as C225 (cetuximab or Erbitux), directed against the extracellular domain of EGFR, which compete with ligand for receptor binding and prevent kinase activation. C225 is currently in phase II and III trials for the treatment of NSCLC (40). Additional antibodies are in clinical development for NSCLC, including the fully humanized monoclonal antibody ABX-EGF (41). The other anti-EGFR agents are the EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib (OSI-774 or Tarceva), which prevent autophosphorylation of EGFR by physical interaction with its intracellular kinase domain (42). Gefitinib is currently available for treatment of advanced NSCLC and erlotinib is in phase III clinical development. Several other EGFR-targeted compounds are in preclinical or early clinical trials for NSCLC treatment (43–45). Together, these data on clinical drug development of both the ER and EGFR pathways support our choice of fulvestrant and gefitinib for lung cancer treatment.

One area of cross-talk that has critical importance in cancers is the interaction between ERs and growth factors. Polypeptide growth factors, such as EGF and insulin-like growth factor-I, stimulate the transcriptional activity of ERs in an estrogen-independent manner. There is evidence that EGF can directly phosphorylate nuclear ERα at Ser118 by phospho-kinase at Ser124 by phospho-p44/p42 MAPK (46). Recent studies suggest that there is also a nonnuclear ER that can activate phosphatidylinositol 3 kinase and the EGFR family of receptors (6). This suggests that in cells that express both EGFR and ER estrogen may stimulate cell proliferation and survival through these alternate pathways and that bidirectional signaling between these two receptors is a potent method of augmenting both estrogen and growth factor action. The rapid responses that we observed in phospho-EGFR and phospho-p44/p42 MAPK suggest that the nonnuclear ER transactivation of EGFR exists in lung cancer cells. Targeting EGFR in combination with traditional hormone therapy could be of benefit by increasing tumor cell death and preventing signaling through this alternate growth pathway.

Targeting both of these pathways is not without precedence and combining therapies to target several aspects of signal transduction should have the most beneficial antitumor effects. Recently, the combination of gefitinib and fulvestrant has been tested in breast cancer cells and an additive effect was observed (25). The addition of an EGFR inhibitor in combination with antihormone therapy in breast cancer patients has been proposed to combat hormone resistance. Breast cancers have also been shown to synthesize estrogens and estrogen is produced locally in tumors from postmenopausal women through the action of aromatase (47). This in situ estrogen production has not been reported in lung tumors. However, estrogen levels are often elevated in female lung cancer patients compared with females without lung cancer (48). In the studies presented here, although the tumor volume in the in vivo xenograft model does not reach statistically significant differences between gefitinib treatment alone and dual treatment, the effect on downstream signaling molecules as well as decreased tumor cellularity and heightened apoptosis suggests that this combination has potential for lung cancer therapy.

EGFR expression in lung tumors does not seem to predict effectiveness of EGFR targeted therapies, as both lung cancer cell lines and primary tissues with low or high EGFR levels were sensitive or insensitive to gefitinib (23, 49). Additionally, with regard specifically to clinical gefitinib sensitivity, Hirsch et al. has shown that EGFR overexpression is most prominent in squamous cell carcinoma and this does not correlate with the clinically observed response pattern to gefitinib: high in adenocarcinoma and low in squamous cell carcinoma (30). The data presented here also show that the squamous cell carcinoma cell line (273T) that we examined was the only one that overexpresses EGFR. This cell line, however, does not show an increased sensitivity to gefitinib in the in vitro studies presented here. Not only does this cell line overexpresses EGFR but it also contains a mutation in the EGFR tyrosine kinase domain. This suggests that not all EGFR mutations produce altered sensitivity to tyrosine kinase inhibitors. Only breast cancer patients with ER-positive tumor status are given fulvestrant treatment. However, no discrepancies have been studied as to ERα or ERβ status as a predictor for response. From the data presented here, the relative expression of ER or EGFR in the tumor does not predict effects on cell lines of treatment with gefitinib or fulvestrant. The discovery that mutations in the tyrosine kinase domain of EGFR predict response to gefitinib in NSCLC patients has enormous impact on identifying patients who will benefit from this treatment (21, 22); however, only a small portion of patients have this mutation. Combination therapy may be especially beneficial in the vast majority of patients whose tumors lack a mutation with functional significance because it potentially provides a second mechanism for inhibiting the EGFR by reducing its transactivation by ERβ.

An inverse correlation between EGFR and ER expression has been well established in breast cancer (6). Treatment of lung cancer cells with fulvestrant gives rise to a population of cells with increased levels of EGFR expression, suggesting that this may be the case in lung cancer as well. The recent evidence of cross-talk between the EGFR and the ER pathways, along with the striking efficacy of gefitinib in women with lung cancer and the emerging role of estrogens in lung tumorigenesis, provides rationale to examine drugs that target these two pathways for lung cancer treatment in selected patients. Because response rates to gefitinib are 10% at best and are almost exclusively found to date in patients with an EGFR mutation, new approaches are needed to increase the efficacy of gefitinib.

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Combined Targeting of the Estrogen Receptor and the Epidermal Growth Factor Receptor in Non–Small Cell Lung Cancer Shows Enhanced Antiproliferative Effects

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