The Effects of Cetuximab Alone and in Combination With Radiation and/or Chemotherapy in Lung Cancer

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ABSTRACT

Purpose: **The epidermal growth factor receptor (EGFR) overexpressed in approximately 80% of non-small cell lung cancers (NSCLC) is a target for novel therapeutics. Concurrent chemoradiation is the current standard of care for treatment of patients with locally advanced NSCLC. However, < 20% of patients remain disease-free at 5 years despite this aggressive treatment. Cetuximab is a humanized monoclonal antibody that recognizes the human EGFR, and in previous studies, inhibited the growth of EGFR-expressing human cancer cell lines. In this report, we investigated the cellular and molecular effects of cetuximab alone and in combination with radiation and/or chemotherapy in human NSCLC cell lines with varying levels of EGFR overexpression *in vitro* and *in vivo*.**

Experimental Design: **We evaluated the EGFR status of a panel of human NSCLC cancer cell lines by immunohistochemistry and flow cytometry. We then evaluated cetuximab effects on growth, cell cycle distribution, and downstream intracellular signaling molecules in this panel of NSCLC cancer cell lines. NSCLC cell lines were treated with cetuximab alone or in combination with radiation, chemotherapy, or chemoradiation to determine the cooperative effects of cetuximab both *in vitro* and *in vivo* in athymic nude mice bearing NSCLC xenografts.**

Results: **Cetuximab alone inhibited the *in vitro* growth of some but not all EGFR-expressing NSCLC cell lines in a dose-dependent manner. Flow cytometric analysis of cell cycle distribution after 24 hours of cetuximab treatment revealed a shift into the G_0/G_1 phase of the cell cycle in cetuximab-sensitive EGFR-expressing cell lines and at concentrations that were growth-inhibitory. There were no cell cycle changes in the EGFR-negative cell lines. After 4 hours of exposure, cetuximab reduced epidermal growth factor (EGF)-induced phosphorylation of EGFR (pEGFR) and HER-2 (pHER2) in cetuximab-sensitive cell lines but not in cetuximab-resistant cell lines. Cetuximab reduced EGF-induced phosphorylation of extracellular signal-regulated kinase-1/2 (pERK) in all EGFR-expressing cell lines. In the absence of EGF, cetuximab alone increased the level of pEGFR and pHER2 above that seen in untreated control cells in both sensitive and resistant cell lines that were EGFR- and HER2-positive, but not in EGFR- or HER2-negative lines. Despite the cetuximab-induced increase in phosphorylation of EGFR and HER2, peak EGF-induced levels of pEGFR and pHER2 were reduced by cetuximab in the cetuximab-sensitive lines but not in the resistant lines. Cooperative (combination index values < 1.0) growth inhibitory effects were observed *in vitro* combination assays with cetuximab and radiation only in cetuximab-sensitive NSCLC cell lines. A lack of cooperation was seen in cetuximab-insensitive NSCLC cell lines. Similar findings were observed with *in vitro* combination studies of cetuximab plus cisplatin or paclitaxel. In nude mice bearing EGFR-expressing, cetuximab-sensitive, NSCLC cell line xenografts, cetuximab plus radiation induced a marked improvement in tumor growth inhibition over either agent alone. The growth inhibitory effects of cetuximab-radiation were similar to the growth inhibitory effects of concurrent chemoradiation. Triple combination therapy of cetuximab and chemoradiation yielded a nonsignificant advantage in tumor growth control over doublet combinations (cetuximab and radiation or chemoradiation) *in vivo*.**

Conclusions: **Similar results in tumor growth inhibition observed in mice treated with cetuximab-radiation and cisplatin-radiation provide a rationale for the clinical investigation of cetuximab with concurrent radiation in selected patients with locally advanced NSCLC. Local tumor control and treatment toxicity should be evaluated between cetuximab-radiation and chemoradiation regimens. Proper patient selection will be critical to the success of such trials and further studies are needed to identify optimal patient selection criteria.**

INTRODUCTION

Lung cancer will claim the lives of 160,440 Americans in 2004 (1). The majority of non-small cell lung cancer (NSCLC) patients present with stage III or IV disease and the majority of such patients cannot be cured by available therapies. NSCLC patients with unresectable stage III disease or medically inoperable disease account for about 40% of all patients diagnosed with NSCLC. Radiation therapy remains the mainstay of treatment in such patients. Chemotherapy, when added

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concurrently to chest radiotherapy, significantly improves the survival of unresectable stage IIA and IIIB patients and is now the treatment of choice (2, 3). However, complete response rates are low and long-term survival remains poor. Targeted biological therapies that selectively interfere with cancer cell and/or endothelial cell growth signaling may improve lung cancer patient survival by enhancing radiation and chemotherapy efficacy without increasing treatment-related toxicity.

One such target is the epidermal growth factor receptor (EGFR), which is widely expressed in NSCLC (4–6). NSCLCs often produce and secrete transforming growth factor-α, amphiregulin, and epidermal growth factor (EGF), which bind to and activate cell surface EGFR (7). Interfering with the EGFR signaling pathway may counteract lung cancer growth. Inhibitors of the EGFR signaling pathway include monoclonal antibodies to the EGFR and small molecule receptor tyrosine kinase inhibitors, which are discussed in great detail in recent reviews (8, 9). Several EGFR-targeted antibodies and receptor tyrosine kinase inhibitors have entered clinical trials in patients with advanced lung cancer. Gefitinib, a receptor tyrosine kinase inhibitor, received Food and Drug Administration approval in May 2003 for use in advanced NSCLC patients refractory to chemotherapy. Under investigation in NSCLC is cetuximab, a chimeric human-mouse monoclonal antibody that binds to the EGFR and inhibits the growth of EGFR-expressing cancer cell lines *in vitro* and *in vivo* in athymic nude mice (10, 11).

Although the effectiveness of biological agents, including EGFR inhibitors, as monotherapy may be limited in patients with advanced cancer, it is rational to consider combining agents such as cetuximab with proven therapies such as radiation. Radiation activates EGFR signaling leading to radioresistance by inducing cell proliferation and enhanced DNA repair (12). In addition, the level of EGFR expression is inversely related to the effectiveness of radiation therapy to control cancer cell growth (13). This inverse relationship has important implications in treatment strategies designed to control EGFR-expressing cancer cell clones that survive radiation. Studies have been undertaken to determine whether blocking EGFR signaling improves radiation cytotoxicity. Additive or synergistic (cooperative) growth inhibition was observed when cetuximab was combined with radiotherapy (and chemotherapy) in several preclinical models (14–18).

Because there is little information on the role of EGFR inhibitors with radiation in NSCLC, we investigated the growth inhibitory effects of cetuximab alone and in combination with radiation and/or chemotherapy in NSCLC cell lines *in vitro* and *in vivo* in athymic nude mice bearing NSCLC cell line xenografts. We evaluated the relationship between EGFR expression and cetuximab-induced effects on cell cycle distribution, activated signal protein expression, and growth inhibition. We found that cetuximab enhanced the effects of radiation and chemotherapy, both *in vitro* and *in vivo* but only in cell lines sensitive to cetuximab alone. In our studies, we observed that in cells that contained no EGFR or HER2 expression, no response was seen to cetuximab *in vitro* or *in vivo*. These studies lay the foundation for further combined treatment studies in locally advanced NSCLC patients, especially those sensitive to cetuximab.

MATERIALS AND METHODS

Cell lines. The NSCLC cell lines, NCI-H292 and NCI-H157 were kindly provided by Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX). The NSCLC lines A549, Calu3, and NCI-H520, were obtained from the American Type Culture Collection (Rockville, MD). The NCI-H332 NSCLC line was kindly provided by Dr. Al Moustafa (National Research Council Canada, Biotechnology Research Institute, Montreal, Quebec, Canada) and the NCI-H358 line was obtained from Dr. Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX). All cell lines were maintained in RPMI media supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) in a humidified incubator with 5% CO_2 except for Calu3, which was maintained in RPMI media with 10% nonheat-inactivated fetal bovine serum.

Chemicals and Cetuximab. Paclitaxel was provided by Bristol Myers Squibb (Princeton, NJ) and cisplatin was obtained from Sigma Chemical, Co., St. Louis, MO. Cetuximab was generously provided by ImClone Systems, Inc. (New York, NY).

EGFR Cell Surface Expression by Flow Cytometry and Immunohistochemistry. Cells (5×10^5) were incubated with cetuximab, or an isotype-matched control monoclonal antibody (human myeloma IgG1, Sigma). The cells were counterstained with goat anti-human IgG-FITC (Southern Biotechnology, Birmingham, AL). All staining was done on ice for 45 minutes followed by three washes in Hanks balanced salt solution + 5% fetal bovine serum. Following staining, the cell fluorescence was measured by flow cytometry (Coulter EPICS-XL-MCL, Hialeah, FL). Using the Coulter software, the percentage of positive cells and the median fluorescence intensity (MFI) was determined. The MFIs were standardized for comparison using the following formula: [(MFI-EGFR) − (MFI-Isotype Control)/(MFI-Isotype Control)].

For immunohistochemical analysis, the cell lines were centrifuged into a cell pellet and embedded in paraffin. Antigen retrieval was done at 95°C in citrate buffer for 40 minutes. The slides were cooled to room temperature for 20 minutes, washed thrice for 3 minutes with Tris-buffer, peroxidase-blocked for 5 minutes, and washed as above. The NSCLC cell lines were then incubated for 30 minutes with the primary antibody, anti-EGFR clone 31G7 (Zymed, San Francisco, CA), followed by the secondary antibody (Visualization Reagent) followed by the substrate-chromogen solution (3,3′-diaminobenzidine) and finally counterstained with hematoxylin. Two pathologists (W. Franklin and F. Hirsch) independently quantified EGFR-specific cell membrane staining as 0 to 3+.

Growth Inhibition of NSCLC Cells by Cetuximab Alone. The growth inhibitory effects of cetuximab alone and the effects of cetuximab with radiation, cisplatin, or paclitaxel were evaluated using a modified tetrazolium salt (MTT) assay (19). In the MTT assay, 1,000 to 2,000 viable cells were plated in 100 μL of growth medium in 96-well plates (Corning, Ithaca, NY). Following an overnight incubation, cetuximab was added in varying concentrations and incubated for 6 to 7 days. The tetrazolium salt was added at a concentration of 0.4 mg/mL to each well following the 6- to 7-day treatment. The plates were incubated with the salt for 4 hours at 37°C. At 4 hours, the medium was aspirated off, leaving the dark blue formazan

product at the bottom of the wells. The reduced MTT product was solubilized by adding 100 μL of 0.2 N HCl in 75% isopropanol, 23% MilliQ water to each well. Thorough mixing was done using a Titertek multichannel pipetman. The absorbency of each well was measured using an automated plate reader (Molecular Devices, Sunnyvale, CA). All experiments were done in triplicate.

Cell Cycle Distribution. The effects of cetuximab alone on cell cycle distribution were determined by flow cytometric analysis. Cells were stained with saponin/propidium iodide/RNase solution and analyzed by fluorescence-activated cell sorting (FACS) using a Coulter EPICS and ModFit software (Verity House software, Topsham, MN).

Western Blot Analysis. NCI-H292, NCI-H322, NCI-H157, and A549 cells were seeded to reach 80% confluency and then were treated for 4 hours with 100 nmol/L of cetuximab alone or followed by a 15-minute incubation with EGF 10 ng/mL. Precleared cellular lysates were separated on a 4% to 12% SDS-PAGE gel and transferred to polyvinylidene difluoride paper. Protein concentration was determined using the DC Microplate Protein Assay (Bio-Rad, Hercules, CA) and 50 μg of protein was loaded for each sample. Immunoblots for phosphorylated proteins were blocked in 3% protease-free bovine serum albumin (fraction V) (ICN, Aurora, OH) for 1 hour and probed with the following antibodies from Cell Signaling Technology (Beverly, MA): α-phospho-Akt (Ser^{473}) antibody 9271, α-phospho-EGFR (Tyr^{1068}) antibody 2234, phospho-EGFR (Tyr^{845}) antibody 2231, phospho-EGFR (Tyr^{992}) antibody 2235, phospho-EGFR (Tyr^{1045}) antibody 2237, α-phospho-p44/42 extracellular signal-regulated kinase (ERK) 1/2 (Thr^{202}/Tyr^{204}) antibody 9101, and α-phospho-HER2 (Tyr877) antibody 2241S. Immunoblots for unphosphorylated proteins were blocked for 1 hour with 10% nonfat dry milk and probed with the following antibodies from Cell Signaling Technology: α-EGFR antibody 2232, α-HER2 antibody 2242, α-Akt antibody 9275, and α-p44/42 ERK 1/2 antibody 9102. The immunoblots were detected by Vistra Western enhanced chemiluminescence-Plus Blotting Kit (Amersham, Piscataway, NJ) and visualized with a Storm 860 Fluorimager (Molecular Dynamics, Sunnyvale, CA).

Growth Inhibition of NSCLC Cells by Cetuximab in Combination Studies with Radiation or Chemotherapy. MTT growth assays as described above were used to evaluate cetuximab in combination with radiation and chemotherapy. Following an overnight incubation, cetuximab and cytotoxic chemotherapy agents or radiation were added in varying concentrations and incubated for 6 to 7 days. Cetuximab was added 24 hours prior to radiation. The results from the combination assays were analyzed using the isobologram combination index method of Chou and Talalay (20). Combination indices (CI) < 0.9 are indicative of synergistic interactions between the two agents, additive interactions are indicated by CIs of 0.9 to 1.0 and a CI of > 1.1 indicates antagonism between the two agents. Cooperative effects were considered as interactions with a CI < 1.0. All experiments were done in triplicate.

***In vivo* NSCLC Mouse Tumor Model Studies.** Athymic nude mice (4 to 6-week-old females) were obtained from the National Cancer Institute (Bethesda, MD). All animal procedures and maintenance were conducted in accordance with the institutional guidelines of the University of Colorado Health Sciences Center. For radiation studies with cetuximab, 2×10^6 human H292 or 5×10^6 H520 NSCLC cells were injected into the flanks of the mice at day 0. Animals were divided into four groups: control, cetuximab, radiation, and cetuximab combined with radiation. Cetuximab was delivered i.p. in 100 μL, twice a week for 2 to 4 weeks at 1 mg/dose depending on the experiment. Control animals were treated i.p. with the same volume of PBS. Radiation was given as a single 8 Gy fraction using a linear accelerator with customized blocking to treat only the flank containing the NSCLC xenograft. In the combination studies of cisplatin and cetuximab with and without radiation, the radiation treatment was fractionated in the H292 xenografts (5 Gy fractions twice a week for 2 weeks) and a single fraction (8 Gy) in the H520 xenografts. Cisplatin was given i.p. at 8 mg/kg twice a week for 2 weeks in both models. Cetuximab was given as above in this series of experiments. Tumor volume measurements were evaluated twice a week in all experiments by caliper and calculated by the formula: $V = 3.14$ (smaller diameter)^2 (larger diameter)/6.

Statistics. A χ^2 test was done to compare the fraction of cells in G_0/G_1 to the S + G_2-M phase of the cell cycle. In the animal experiments, the mean difference in tumor volumes were compared among treatment groups at the time treatment began, the day treatment stopped, and the last day of tumor measurements using a one-way ANOVA and adding random animal effects.

RESULTS

EGFR Expression in NSCLC Cell Lines. FACS and immunohistochemical analysis characterized a panel of NSCLC cell lines of various histologies for EGFR cell surface expression. The fraction of cells staining positive for EGFR expression by FACS ranged from 0% to 100%. The majority of cell lines (71%) had ≥90% of cells staining positively for EGFR. The MFI of EGFR cell membrane staining by FACS, ranged from 0 to 21. Figure 1*A-D* and Table 1 shows the results of the immunohistochemical and FACS analyses. By FACS analysis, five cell lines had an MFI >8 and ≥90% of the cell population staining positive for EGFR and all were 3+ for EGFR expression by immunohistochemistry. The intensity of EGFR staining by FACS was greater in H292 cells as compared with H332, A549, H157, and Calu3 cells. One NSCLC cell line in our panel, H520, was negative for EGFR expression in both assays. The H358 cell line had the lowest EGFR expression by FACS (89% EGFR+, MFI 4.8).

Effects of Cetuximab on the Growth of NSCLC Cell Lines *In vitro*. Using MTT assays, we evaluated the growth inhibitory effects of cetuximab alone on this panel of NSCLC cell lines. As shown in Fig. 2, 100 nmol/L cetuximab treatment significantly inhibited the growth of the H292 and H332 cell lines, which had the greatest EGFR expression (MFI 21 and 16, respectively) and the Calu-3 line, which had intermediate EGFR expression (MFI, 8.9). The growth inhibitory effects of cetuximab on the A549 (MFI 14) and H358 (MFI 5.7) lines were more moderate. Cetuximab failed to inhibit the growth of H157 cells whose level of EGFR expression (MFI 13) was similar to A549. As expected, cetuximab treatment did not inhibit the

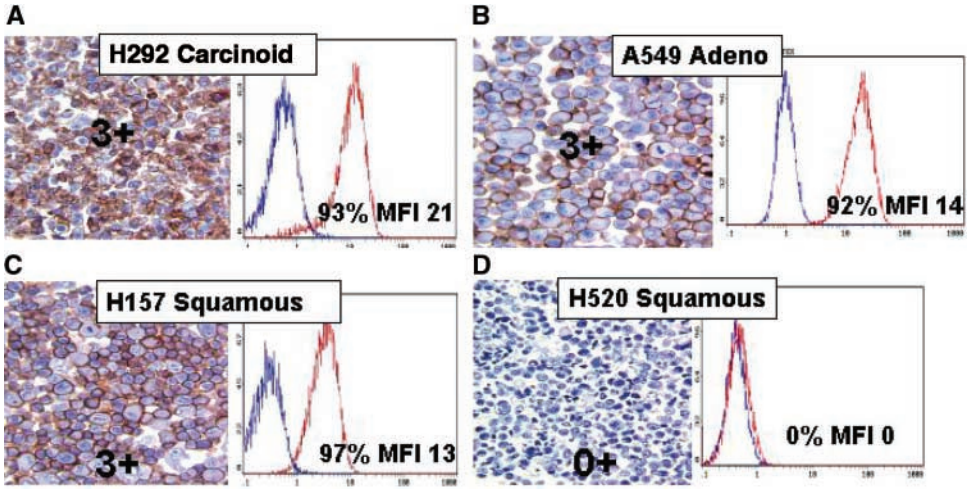

Fig. 1 A-D, expression of EGFR in four NSCLC cells lines as assessed by immunohistochemistry (*left*) and FACS (*right*). Blue peak, staining with the isotype-matched control antibody; red peak, cell surface staining with the anti-EGFR antibody. *A-C,* percentage of positive staining cells for EGFR expression, the MFI, and the immunohistochemical score in three EGFR-positive cell lines. *D,* EGFR-negative cell line.

growth of the EGFR-negative cell line H520. Thus, EGFR expression did not always correlate with growth inhibition.

***In vitro* Effect of Cetuximab on Cell Cycle.** Flow cytometric analysis of the effects of cetuximab alone on cell cycle distribution revealed a statistically significant shift ($P < 0.0001$) into the G_0/G_1 phase of the cell cycle in all five of the EGFR-expressing cell lines that were growth-inhibited by cetuximab (Table 2). The nonresponsive line H157 had no change in cell cycle distribution. The H292 cells with the highest EGFR expression (MFI 21) had an increase in the G_0/G_1 fraction from 35% to 66% shift after a 24-hour cetuximab exposure. The H322 cells (MFI 16) had an increase in G_0/G_1 from 43% to 55%. The more moderately expressing cell line, A549 (MFI 14) had an increase in G_0/G_1 from 49% to 55%. The H157 cells with EGFR expression (MFI 13) similar to the A549 (MFI 14) cells had no change in cell cycle distribution. The low EGFR expression cell lines Calu-3 (MFI 8.9) and H358 (MFI 5.7) had an increase in G_0/G_1 from 50% to 55% and from 31% to 39%, respectively. There were no changes in cell cycle distribution in the NSCLC cell line H520 that lacked EGFR expression, as shown in Table 2.

Cetuximab Effects on EGF-Stimulated EGFR Signaling. We investigated whether downstream signaling pathways were modulated in the cetuximab-responsive (H292, H332), moderately responsive (A549), and nonresponsive (H157) NSCLC cell lines that express EGFR and in the EGFR null line H520 by treating the cells with cetuximab alone and cetuximab followed by EGF. To assess the effects of cetuximab on EGF-induced signaling, the cell lines were incubated with 100 nmol/L cetuximab for 4 hours followed by EGF 10 ng/mL for 15 minutes. Inhibition of EGF-induced EGFR (Tyr^{1068}) autophosphorylation was observed in all EGFR-expressing cell lines but to a greater degree in the cetuximab-sensitive cell lines H292 and H322, and the moderately cetuximab-sensitive line A549 (Fig. 3*A*). The least inhibition of EGF-induced EGFR autophosphorylation was observed in the cetuximab-resistant cell line, H157 (Fig. 3*A*). In the cetuximab-sensitive lines, H292 and H322, cetuximab exposure produced strong inhibition of EGF-induced HER-2 (Tyr^{1248}) phosphorylation (Fig. 3*B*). Modest inhibition of EGF-induced HER-2 phosphorylation was observed in the A549 and H157 cell lines (Fig. 3*B*). No endogenous HER-2 expression was detected by Western immunoblotting in the H520 cell line, which is in agreement with the FACS and immunohistochemical analysis of this line (Fig. 3*B*). EGF-induced ERK 1/2 ($Thr^{202}Tyr^{204}$) protein phosphorylation was moderately inhibited by cetuximab treatment in all cell lines except the non-EGFR-expressing line H520 (Fig. 3*C*). In summary, we observed a greater degree of inhibition of EGF-induced phosphorylation by cetuximab of both EGFR and HER-2 in the H292 and H322 cell lines, inhibition of EGF-induced ERK 1/2 phosphorylation by cetuximab seemed similar in all the EGFR-expressing cell lines. Despite EGF stimulation, no significant alterations in pAkt were observed in the same panel of NSCLC cell lines when treated with cetuximab (Fig. 3*D*).

Effects of Cetuximab Alone on EGFR Signaling. We also investigated the effects of cetuximab alone without EGF stimulation in the H292, H322, A549, H157, and H520 cell lines as seen in Fig. 3*A-D*. As expected, cetuximab (4-hour incubation) had no effect on EGFR downstream signaling in the EGFR and HER2 null cell line H520 (Fig. 3*A-C*). Surprisingly, cetuximab alone increased the level of p-EGFR (Tyr^{1068}) and p-HER2 (Tyr^{1248}) in the H292, H322, A549, and H157 cell lines above the level of p-EGFR and p-HER-2 seen in

Table 1 EGFR expression by flow cytometry and immunohistochemistry in NSCLC cell lines

Cell lines	% EGFR+	MFI	Immunohistochemical score
Adenocarcinoma			
A549	99	14	3+
Calu-3	98	8.9	3+
BAC			
H322	100	16	3+
H358	89	4.8	ND
Squamous			
H157	93	13	3+
H292	100	21	3+
H520	0	0	0

Abbreviations: BAC, bronchoalveolar carcinoma; ND, not done.

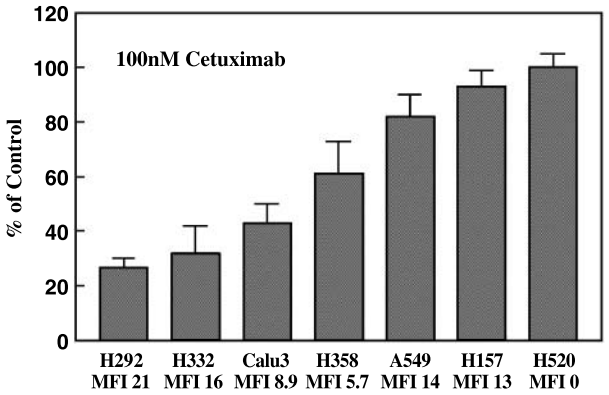

Fig. 2 Growth inhibitory effects of a single administration of 100 nmol/L cetuximab on a panel of NSCLC cell lines with high, moderate, low, and no EGFR expression using MTT assays. *Columns,* mean ± SE of two to three experiments. No growth inhibition was observed in the EGFR-null cell line H520. In the EGFR-expressing cell lines, growth inhibition did not always correlate with EGFR expression level.

the untreated controls (Fig. 3*A* and *B*). In the cetuximab-sensitive cell lines, H292 and H322, p-ERK 1/2 levels were reduced by cetuximab alone compared with control levels and to EGF alone–induced levels (Fig. 3*C*). In the cetuximab moderately sensitive cell line, A549, and the resistant line, H157, cetuximab alone had no effect on the level of pERK 1/2 when compared with controls (Fig. 3*C*). There seemed to be no effects upon p-Akt in the unstimulated panel of NSCLC lines when treated with cetuximab at 100 nmol/L (Fig. 3*D*).

As shown in Fig. 4, cetuximab alone also increased the level of p-EGFR at other tyrosine residues above control levels in the cetuximab-sensitive cell line H322. Cetuximab increased the level of p-EGFR at Tyr^{845}, Tyr^{992}, and Tyr^{1068} above control levels. Cetuximab alone had no effect on p-EGFR Tyr^{1045} a major docking site for c-Cbl, which results in receptor ubiquitination and degradation. EGF induced significant phosphorylation at Tyr^{1045} compared with both control and cetuximab-treated cells.

Effects of Cetuximab in Combination with Radiation, Cisplatin, or Paclitaxel on Cell Growth. The effects of cetuximab in combination with single fraction ionizing radiation were examined using MTT assays (Fig. 5*A-D*). The results were analyzed using the isobologram combination index method of Chou and Talalay (20). Strong synergistic (CIs < 0.5) growth inhibition was observed in MTT assays between cetuximab (3-100 nmol/L) and radiation (2-6 Gy) in cetuximab-sensitive cell lines H292 (MFI 21), and H322 (MFI 16; Fig. 5*A* and *B*). Strong synergy with radiation was also seen in the less responsive lines, Calu-3 (MFI 8.9) and H358 (MFI 5.7; data not shown). Concentrations of 30 to 120 nmol/L cetuximab produced synergistic interactions (CI, 0.66-0.84) with 2 to 6 Gy radiation in the moderately cetuximab-sensitive NSCCL cell line A549 (MFI 14; data not shown). Additive effects (CI, 0.86-1.0) were seen with all combinations of radiation (2-6 Gy) and cetuximab (30-120 nmol/L) in the H157 (MFI 13) cell line, which showed no response to cetuximab alone (Fig. 5*C*). We observed additive to antagonistic effects between cetuximab and radiation at any concentration evaluated in the negative EGFR and cetuximab-insensitive cell line H520 (CI, 0.96-1.2; Fig. 5*D*).

Table 3 summarizes the CIs from cetuximab (6-60 nmol/L) in combination with cisplatin and paclitaxel. Similar to enhanced radiation response, enhanced response to cisplatin (IC_{30-50}) was greater in the cell lines with the greatest response to cetuximab alone. Strong synergistic interactions occurred between cisplatin and all concentrations of cetuximab in the H322 (MFI 16) and Calu-3 (MFI 8.9) cell lines (CI, 0.38 and 0.03, respectively). Synergy to moderate synergy occurred between cisplatin and all concentrations of cetuximab tested in the H292 (MFI 21), and H358 (MFI 5.7) cell lines (CI, 0.68 and 0.73, respectively). Modest synergy was noted between cisplatin and cetuximab in the A549 (MFI 14) cell line (CI = 0.85). In the cetuximab-resistant H157 (MFI 13) cell line, the interactions between cisplatin and cetuximab were predominantly antagonistic (CI = 1.1). The interactions between cisplatin and cetuximab were antagonistic in the EGFR-negative H520 cell line (CI = 1.2).

Synergistic interactions (CI = 0.5) were observed between paclitaxel (IC_{60}) and cetuximab in the low EGFR-expressing cell line Calu-3 (MFI 8.9). Moderate synergy was observed between paclitaxel (IC_{60-80}) and cetuximab in the H292 (MFI 21, CI = 0.63), H322 (MFI 16, CI = 0.71), H358 (MFI 5.7, CI = 0.71), and A549 (MFI 14, CI = 0.7). Little interaction was observed between paclitaxel (IC_{70}) and cetuximab in the H157 cell line (CI = 1.0) or in the EGFR null line H520 (IC_{70}) (CI = 0.92).

***In vivo* Combination Treatment with Cetuximab and Radiation in NSCLC Xenografts.** Based on the *in vitro* studies, we evaluated the effects of cetuximab (i.p.) in combination with single fraction radiation on H292 (EGFR-MFI 21) and H520 (EGFR-MFI 0) tumors propagated in the flanks of athymic nude mice. Treatment began on day 22 when the H292 tumors had reached 3 cm^3. On day 60,

Table 2 The effects of cetuximab on cell cycle distribution in a panel of NSCLC cell lines exposed to 100 nmol/L for 24 hours

Cell line	MFI	% G_0/G_1	% S	% G_2-M	P^*
H292-Squamous	21				
0 nmol/L C225		35	36	29	
100 nmol/L C225		66	20	14	< 0.0001
H322-BAC	16				
0 nmol/L		43	32	25	
100 nmol/L		55	24	21	< 0.0001
H358-BAC	5.7				
0 nmol/L		31	51	18	
100 nmol/L		39	44	17	< 0.0001
A549-Ad	14				
0 nmol/L C225		49	37	14	
100 nmol/L C225		55	35	10	< 0.0001
Calu3-Adenocarcinoma	8.9				
0 nmol/L		50	42	8	
100 nmol/L		55	37	8	< 0.0001
H157-Squamous	13				
0 nmol/L C225		55	30	15	
100 nmol/L C225		55	28	17	N.S.
H520-Squamous	0				
0 nmol/L C225		41	41	18	
100 nmol/L C225		41	42	17	N.S.

Abbreviation: N.S., not significant.
*Per χ^2 test.

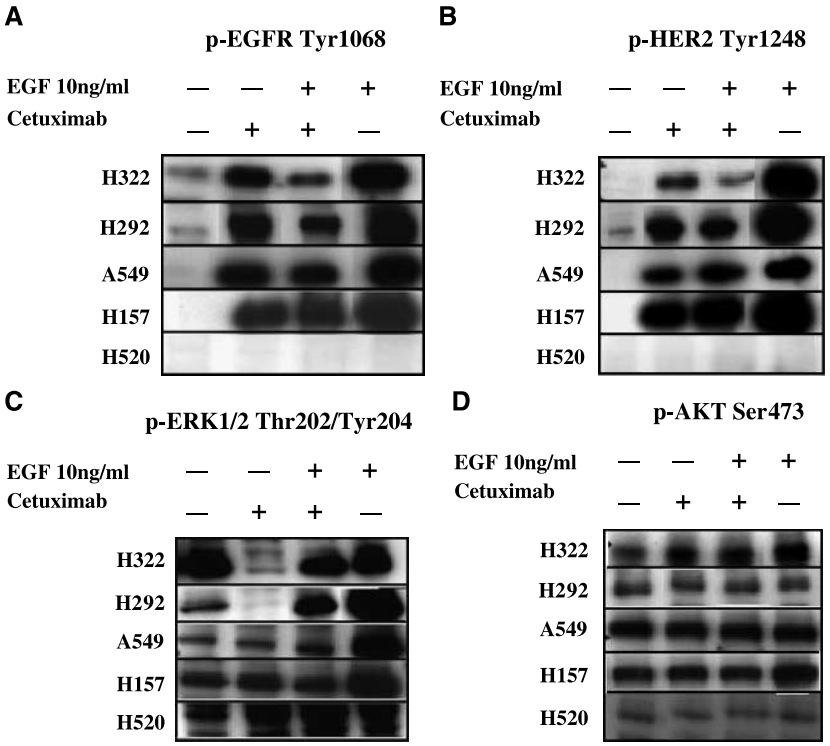

Fig. 3 A-D, effects of cetuximab alone and on EGF-induced HER-2/*neu*, EGFR, ERK 1/2 and AKT phosphorylation in human NSCLC cell lines H332, H292, A549, H157 and H520. Cells were treated for 4 hours with cetuximab alone or followed by EGF for 15 minutes prior to harvesting. Precleared cellular lysates were separated (50 μg of protein was loaded for each sample) on a 4% to 12% SDS-PAGE gel and transferred to polyvinylidene difluoride paper. Immunoblots were blocked in 3% protease-free bovine serum albumin (fraction V) for 1 hour at room temperature and probed with the relevant antibodies. The immunoblots were visualized with enhanced chemiluminescence.

post-tumor cell implantation, cetuximab alone (1 mg i.p. twice a week for 4 weeks) modestly delayed H292 tumor growth over vehicle control (3.5 versus 5.3 cm^3, respectively). The differences in tumor volume were not significant, as shown in Fig. 6*A* and Table 4. Single fraction radiation alone (8 Gy/week for 2 weeks) produced a slight growth delay but again the differences in H292 tumor growth were not significantly different from controls (5.1 versus 5.3 cm^3, respectively). Cetuximab + 8 Gy radiation reduced H292 tumor size on day 60 (1.8 cm^3) compared with controls (5.3 cm^3) and 8 Gy radiation alone (5.1 cm^3) or cetuximab alone (3.5 cm^3). However, the difference was only significant for cetuximab + RT versus control ($P = 0.005$) and cetuximab + RT versus radiation alone ($P = 0.015$).

In the EGFR-null H520 xenografts, cetuximab treatment began on day 18 when the tumors reached 3 cm^3 and continued for 2 weeks (1 mg i.p. twice a week). On day 42 post-tumor cell implantation, the mean tumor volume in the cetuximab-treated H520 tumors were higher then the mean tumor volume of the control group (3.7 versus 2.9 cm^3; Fig. 6*B*; Table 5). However, the differences were not statistically significant ($P = 0.50$). In this EGFR-null line, cetuximab + radiation did not significantly enhance tumor growth control over radiation alone (2.1 versus 2.6 cm^3, $P = 0.70$).

***In vivo* Combination Treatment with Cetuximab, and Concurrent Chemoradiation in NSCLC Xenografts.** We evaluated whether cetuximab (1 mg i.p. twice a week for 4 weeks) plus fractionated radiation (5 Gy twice a week for 2 weeks) would provide similar tumor growth inhibition to cisplatin (8 mg/kg i.p. twice a week for 2 weeks) plus fractionated radiation (standard therapy in NSCLC), and whether the triple combination of adding cetuximab to cisplatin-fractionated radiation would provide optimal tumor growth delay. Treatment began on day 31 when H292 tumors had reached 3 cm^3. On day 93 post-tumor cell implantation, fractionated radiation alone produced a statistically significant reduction in H292 tumor volume compared with controls (2.2 versus 4.4 cm^3, respectively, $P = 0.049$; Fig. 7*A*; Table 6). Fractionated radiation plus cisplatin also produced a statistically significant reduction in H292 tumor volume compared with controls (1.8 versus 4.4 cm^3, $P = 0.024$). As anticipated, cetuximab in combination with fractionated radiation also produced a statistically significant reduction in H292 tumor volume compared with controls (1.5 versus 4.4 cm^3, $P = 0.012$). The combination of all three modalities, cetuximab, radiation, and cisplatin, produced a somewhat great growth delay (1.2 cm^3) compared with the double combination of cetuximab plus radiation (1.5 cm^3, $P = 0.79$), or cisplatin plus radiation (1.8 cm^3, $P = 0.54$), but these differences were not significant (Table 6). We observed no increased toxicity with the triple combination.

In the H520 xenografts, treatment began on day 18 when the tumor reached 3 cm^3. On day 42, the growth inhibition in mean tumor volume for the EGFR-null H520 xenografts treated with standard therapy cisplatin (8 mg/kg i.p. twice a week for 2 weeks) + radiation (8 Gy single fraction) was greater but not significantly than that seen in the controls (0.65 versus 2.9 cm^3, $P = 0.07$). Tumor growth in cetuximab (1 mg i.p. twice a week) + radiation-treated animals was not significantly different from control tumor growth (2.1 versus 2.9 cm^3, $P = 0.55$) (Fig. 7*B*; Table 5). The triple therapy of cetuximab + radiation + cisplatin was also less effective at inhibiting tumor growth then the combination of cisplatin + radiation (1.1 versus 0.65 cm^3, $P = 0.73$).

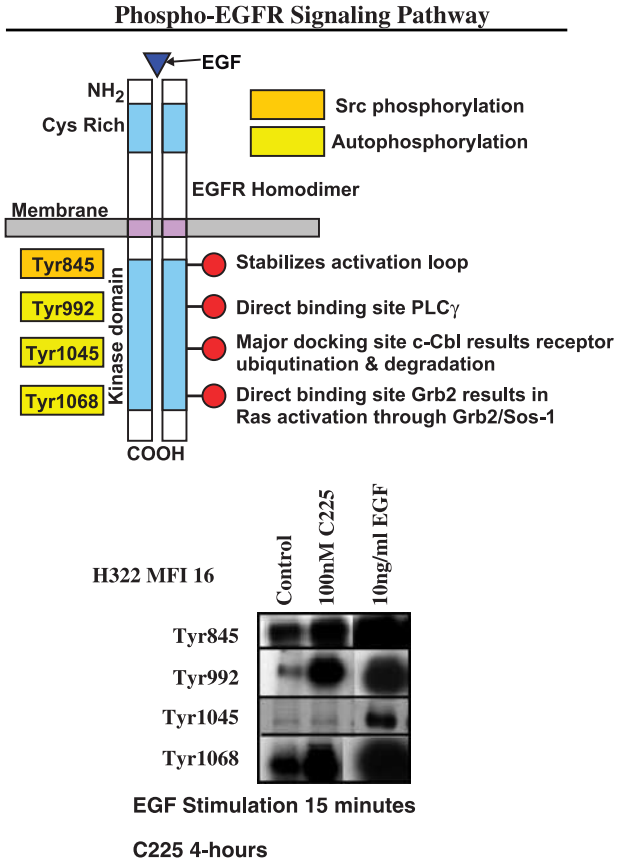

Fig. 4 The effects of 4-hour cetuximab treatment alone versus 15-minute treatment of EGF alone on EGFR phosphorylation sites involved in downstream signaling pathways in the NSCLC cell line H322. Cells were treated for 4 hours with cetuximab followed by EGF for 15 minutes prior to harvesting. Precleared cellular lysates were separated (50 μg of protein was loaded for each sample) on a 4% to 12% SDS-PAGE gel and transferred to polyvinylidene difluoride paper. Immunoblots were blocked in 3% protease-free bovine serum albumin (fraction V) for 1 hour at room temperature and probed with the relevant antibodies shown. The immunoblots were visualized with enhanced chemiluminescence.

DISCUSSION

Previous studies have shown that NSCLCs frequently have high expression of the EGFR as well as its ligands EGF and transforming growth factor-α (7). Some of these studies suggested that increased expression of EGFR was associated with a worse prognosis in stage IV patients but this was not universally observed (5, 21–25). We found that EGFR expression by immunohistochemistry or fluorescence *in situ* hybridization did not correlate with survival (26). In addition, mutations and/or true gene amplification of the EGFR gene in NSCLC are uncommon.

Inhibitors of EGFR, such as the monoclonal antibody cetuximab, were shown to inhibit the growth of some EGFR over-expressing cell lines of various histologies including NSCLC both *in vitro* and *in vivo* (8–11). Other studies indicated that combinations of cetuximab with radiation and chemotherapeutic agents produced synergistic growth inhibition in EGFR-expressing and cetuximab-sensitive cell lines both *in vitro* and *in vivo* (12, 27–31). The mechanism of growth inhibition seemed to be the inhibition of EGF-induced downstream signaling pathways. Although there are no published single agent data with cetuximab in advanced NSCLC, recent clinical studies indicated that gefitinib produced objective responses in a small minority of advanced chemorefractory NSCLC patients (32, 33). In these NSCLC patients, response did not correlate with EGFR expression level, and combinations of chemotherapy and gefitinib or erlotinib did not improve outcome over chemotherapy alone in untreated stage IV NSCLC patients (34, 35). What do our studies add to this information?

Our studies showed that cetuximab inhibited the growth of some, EGFR-expressing cell lines and that cetuximab sensitivity did not clearly correlate with EGFR expression levels. In our studies, cetuximab failed to inhibit the growth of EGFR-null cell lines. In cetuximab-sensitive cell lines, changes in EGF-induced activated cell signal protein expression were noted at 4 hours after exposure. These early changes in phosphorylation of activated cell signal proteins were followed by a G_1 arrest that was apparent at 24 hours and lasted through at least 72 hours. These changes appeared only in cell lines that responded to cetuximab.

EGFR expression did not predict changes in phosphorylation states, G_1 arrest, or growth inhibition, but there was a correlation between changes in phosphorylation of signal proteins, G_1 arrest, and growth inhibition. For example, the cell lines H322 and A549 have similar expression levels of EGFR, but cetuximab produced greater changes in signal protein phosphorylation, G_1 arrest and growth inhibition in H322 cells then in the A549 cells. H322 cells express the HER-2 receptor in addition to the EGFR, whereas the A549 line has extremely low HER2 expression. Cetuximab inhibited EGF-induced phosphorylation of HER2 as well as EGFR in the H322 cells but to a lesser degree in the A549 cells. Thus, expression of other erbB family receptors may be one factor affecting sensitivity to EGFR inhibitors.

Other factors affecting sensitivity to EGFR inhibitors may include autocrine production of EGF and transforming growth factor-α, or mutations in or expression levels of downstream signaling proteins such as *ras*, PTEN, Akt, or STAT-3. Recent studies seem to corroborate the latter hypothesis. Persistent signaling of either the mitogen-activated protein kinase or phosphatidylinositol 3′-kinase/Akt pathways or both were observed in serum-starved NSCLC cell lines treated with either cetuximab or gefitinib for 2 hours followed by EGF for 5 minutes (36). The moderately cetuximab-sensitive cell lines H358 and A549 and the cetuximab-resistant line H157 have a mutant *ras* gene, whereas the cetuximab-sensitive line H322 has wild-type *ras* suggesting wild-type *ras* may also play a role in cetuximab sensitivity (ATCC.org). PTEN expression however, does not seem to account for differences in cetuximab sensitivity, as H157 (cetuximab-resistant) does not express PTEN, whereas both H292 (cetuximab-sensitive), and A549 (moderate cetuximab sensitivity) express PTEN (37). Additional studies to assess the effect of *ras* mutations and PTEN expression are needed. We have insufficient data from these cell lines to determine which of these factors, if any, plays the most important role in predicting sensitivity to EGFR inhibitors.

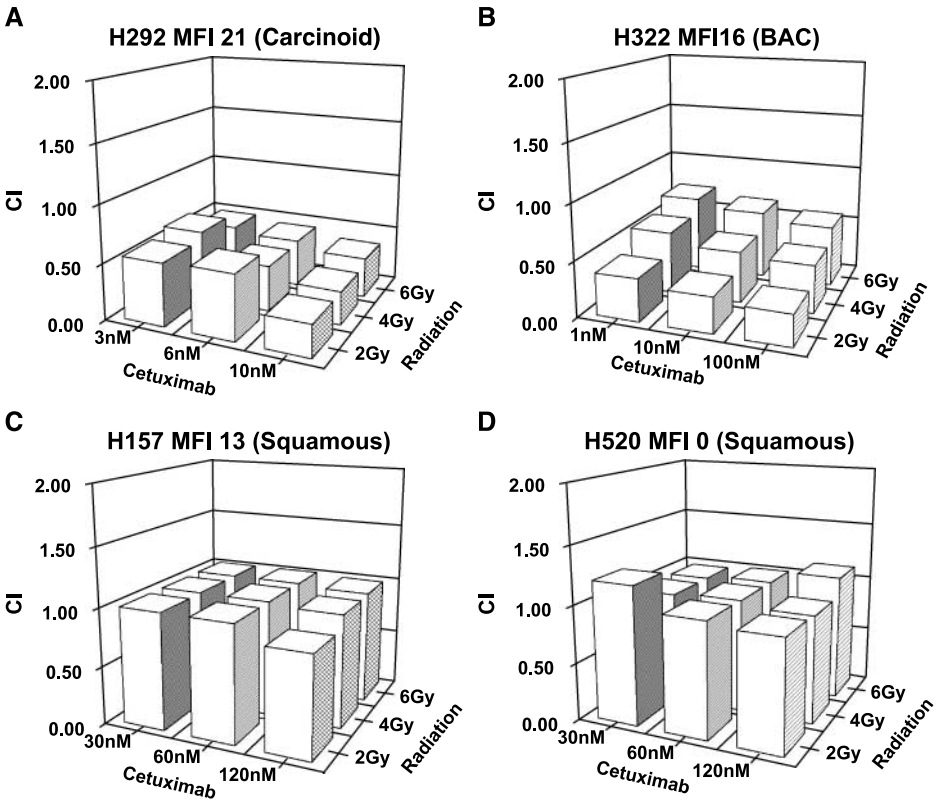

Fig. 5 A-D, CI in studies of cetuximab (3-120 nmol/L) combined with 2 to 6 Gy of irradiation. Cetuximab was added 24 hours prior to irradiation. The results were analyzed after 7 days using the isobologram combination index method of Chou and Talalay (20). CIs < 0.9 are indicative of synergistic interactions between the two agents, additive interactions are indicated by CIs of 0.9 to 1, and a CI of >1 indicates antagonism between the two agents. Strong synergy was seen between cetuximab and radiation in H226 and H322 cells (*A* and *B*). Additive interactions were seen in H157 cells (*C*). Additivity to antagonism occurred between cetuximab and radiation in the EGFR-negative and cetuximab-insensitive H520 cell line (*D*).

We found that cetuximab alone enhanced the level of basal phosphorylation of EGFR and HER2 in EGFR-expressing NSCLC lines. This enhancement of basal phosphorylation of EGFR was not observed with gefitinib treatment (data not shown). The phosphorylation of the EGFR induced by cetuximab alone was still associated with a G_1 arrest and growth inhibition in cetuximab-sensitive cell lines. Therefore, cetuximab did not induce normal EGF-induced survival signaling. Furthermore, cetuximab inhibited EGF-induced signaling as described above in RESULTS.

Prior studies indicated that the combination of cetuximab with radiotherapy produced synergistic growth inhibition in cell lines of other tumor types (14–18). Invariably, these studies were done in cetuximab-sensitive cell lines. Our studies reported here show that the combination of cetuximab with radiation produces cooperative interactions (additivity, CI, 0.9-1; or synergy, CI, < 0.9) in cetuximab-sensitive cell lines such as H292 and H322, and in the moderately cetuximab-sensitive line A549. Synergy was also seen with radiation in the cetuximab-sensitive line Calu3 and in the moderately sensitive line H358 (data not shown). Despite the fact that H157 does not respond well to cetuximab alone, some evidence for additivity (CI, 0.86-1.0) was observed in combination with cetuximab and radiation. Additivity to antagonism (CI, 0.96-1.2) was noted in the H520 cell line, which neither expresses EGFR nor responds to cetuximab.

The combination results between cetuximab and cisplatin or paclitaxel were similar in pattern and magnitude. Synergistic to moderately synergistic interactions were observed with chemotherapy in the cetuximab-sensitive cell lines (H292, H322, and Calu3) and in the moderately cetuximab-sensitive cell lines (H358, A549) and additivity to antagonism was observed in the cetuximab-resistant cell lines (H157, H520).

Our studies indicated similar tumor growth inhibition between animals bearing cetuximab-sensitive NSCLC xenografts

Table 3 CIs for cetuximab + chemotherapy

CIs of cetuximab + cisplatin in NSCLC cell lines

Cell line	CDDP (μmol/L)	MFI	Average
Calu3	0.6 (IC_{50})	8.9	0.030
H322	0.3 (IC_{30})	16	0.38
H292	1 (IC_{40})	21	0.68
H358	0.3 (IC_{40})	5.7	0.73
A549	1 (IC_{30})	14	0.85
H157	0.4 (IC_{30})	13	1.1
H520	1 (IC_{30})	0	1.2

CIs of cetuximab + paclitaxel in NSCLC cell lines

Cell line	P (μmol/L)	MFI	Average
Calu3	10 (IC_{60})	8.9	0.50
H292	3 (IC_{70})	21	0.63
A549	8 (IC_{60})	14	0.7
H322	6 (IC_{60})	16	0.71
H358	3 (IC80)	5.7	0.71
H520	6 (IC_{70})	0	0.92
H157	4 (IC_{70})	13	1.0

Abbreviations: CDDP, cisplatin; IC, inhibitory concentration; P, paclitaxel; IC, inhibitory concentration.

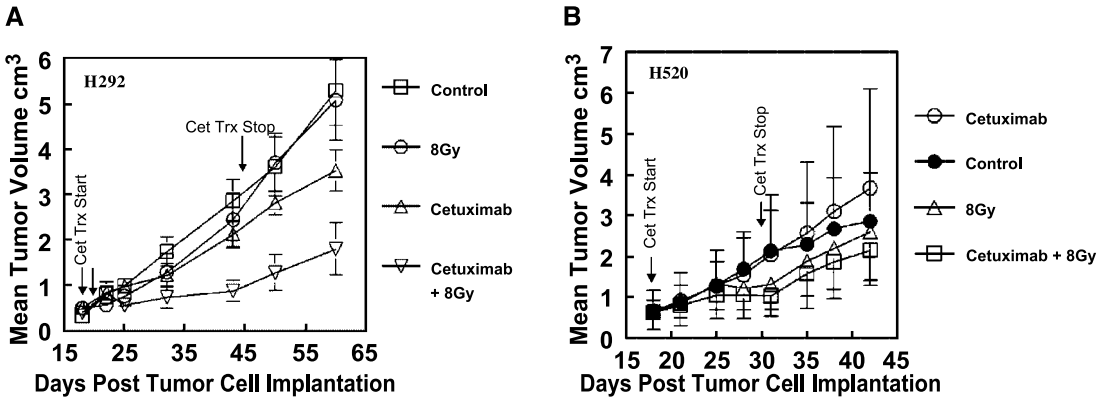

Fig. 6 *A-B,* effects of cetuximab combined with radiation on H226 (EGFR-positive) and H520 (EGFR-negative) NSCLC xenografts in athymic mice. Cetuximab treatment began when tumors had reached approximately 3 cm^3. Radiation treatment began a day later. Each treatment group contained a minimum of five mice. Results shown indicate mean tumor volumes ± SE. Single dose radiation (8 Gy) had a minimal and nonsignificant effect on tumor growth in both the H292 and H520 xenografts. Cetuximab given i.p. at 1 mg/animal twice a week for 4 weeks reduced day 60 tumor volume from 5.3 to 3.5 cm^3 in the H292 xenografts. This difference was of borderline significance ($P = 0.1$). The combination of radiation + cetuximab reduced day 60 H292 tumor volume from 5.3 in controls to 1.8 cm^3. This reduction was statistically superior to controls ($P = 0.005$) and to radiation alone ($P = 0.015$), and marginally superior to cetuximab alone ($P = 0.16$). In the H520 xenografts, cetuximab alone and in combination with radiation did not inhibit tumor growth.

treated with cetuximab-radiation and cisplatin-radiation. No tumor growth inhibition was observed in mice bearing cetuximab-insensitive xenografts. Therefore, it may be reasonable to consider clinical trials that compare targeted therapy in combination with radiation to conventional chemoradiation regimens with regards to toxicity (which can be substantial in chemoradiation regimens) and response rates.

Our preclinical results indicate that it could be beneficial to combine cetuximab with standard therapy regimens for NSCLC for some patients but detrimental in other patients. It is possible that these observations could account for the negative results in the INTACT 1-2 trials and a recent trial comparing erlotinib and chemotherapy to chemotherapy alone (34, 35). In contrast to the negative chemotherapy + EGFR-TKIs trials sited above, recent combination clinical trials of cetuximab and docetaxel showed promising response rates in chemorefractory NSCLC patients (40). Phase III studies of cetuximab and concurrent chemotherapy as first line therapy in EGFR-positive advanced stage NSCLC patients also showed positive response benefits over chemotherapy alone (41). Based on positive phase III trial outcomes in patients with advanced, chemorefractory colon cancer, cetuximab has recently been approved by the Food and Drug Administration for treatment with concurrent chemotherapy in patients with advanced colon cancer.

From a radiation therapy standpoint, cetuximab combined with radiation showed encouraging response rates with no significant increase in toxicity in a phase I study in locally advanced head and neck cancer (42). Results from a completed phase III trial comparing radiation alone to radiation plus cetuximab in head and neck cancer are pending.

The studies presented in this manuscript suggest that it is critical to give combination cetuximab chemo or radiation therapy to a *select* group of patients and that selection of patients for clinical trials based on EGFR expression alone may not be helpful. The need for preselection of patients based on biologic/genetic factors that predict sensitivity/resistance is likely to be true for combinations of cetuximab

Table 4 Effects of cetuximab + single fraction radiation (8 Gy) on mean tumor volume in athymic nude mice bearing NCI-H292 tumors

Treatment	Tumor volume, day 60 (cm^3)	*P*
Cet vs. Control	3.5 ± 1.0 vs. 5.3 ± 2.4	0.10
RT vs. Control	5.1 ± 2.0 vs. 5.3 ± 2.4	0.80
Cet vs. RT	3.5 ± 1.0 vs. 5.1 ± 2.0	0.20
Cet + RT vs. Control	1.8 ± 1.3 vs. 5.3 ± 2.4	0.005
Cet + RT vs. Cet	1.8 ± 1.3 vs. 3.5 ± 1.0	0.16
Cet + RT vs. RT	1.8 ± 1.3 vs. 5.1 ± 2.0	0.015

Abbreviations: Cet, cetuximab; RT, radiation.

Table 5 Effects of cetuximab + single fraction radiation (8 Gy) and/or cisplatin on mean tumor volume in athymic mice bearing NCI-H520 tumors

Treatment	Tumor volume, day 42 (cm^3)	*P*
Cet vs. Control	3.7 ± 2.4 vs. 2.9 ± 1.2	0.50
RT vs. Control	2.6 ± 1.3 vs. 2.9 ± 1.2	0.83
Cet vs. RT	3.7 ± 2.4 vs. 2.6 ± 1.3	0.38
Cet + RT vs. Control	2.1 ± 0.73 vs. 2.9 ± 1.2	0.55
Cet + RT vs. Cet	2.1 ± 0.73 vs. 3.7 ± 2.4	0.21
Cet + RT vs. RT	2.1 ± 0.73 vs. 2.6 ± 1.3	0.70
CDDP + RT vs. Control	0.65 ± 0.35 vs. 2.9 ± 1.2	0.07
Cet + RT + CDDP vs. Control	1.1 ± 0.55 vs. 2.9 ± 1.2	0.14
Cet + RT + CDDP vs. Cet + RT	1.1 ± 0.55 vs. 2.1 ± 0.73	0.37
Cet + RT + CDDP vs. CDDP + RT	1.1 ± 0.55 vs. 0.65 ± 0.35	0.73

Abbreviations: Cet, cetuximab; RT, radiation; CDDP, cisplatin.

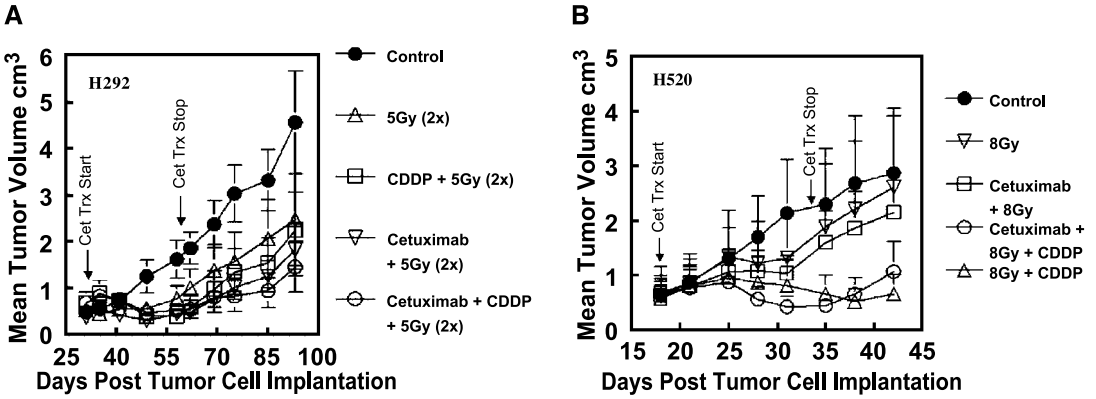

Fig. 7 *A-B,* effects of cetuximab combined with radiation and cisplatin on human H292 and H520 NSCLC xenografts in athymic nude mice. Treatment began when tumors had reached approximately 3 cm^3. Treatments were cetuximab alone (1 mg/animal twice a week for 2 weeks), ionizing radiation (5 Gy each Monday for 2 weeks), cisplatin (8 mg/kg twice a week for 2 weeks), and combinations of cetuximab plus radiation, cisplatin plus radiation, and cetuximab plus cisplatin plus radiation treatments. Tumor volume ± SE are shown. On day 93, the H292 mean tumor volume was 4.4 cm^3 in control animals, 2.2 cm^3 in animals treated with irradiation alone ($P = 0.049$), 1.8 cm^3 in animals treated with cisplatin plus radiation ($P = 0.024$), 1.5 cm^3 in animals treated with cetuximab plus radiation ($P = 0.012$), and 1.2 cm^3 in animals treated with cetuximab plus cisplatin and radiation ($P = 0.004$). The combinations produced a significant reduction in tumor volume compared with controls. Although the combination of all three agents produced the greatest reduction in tumor volume, the differences were not significant compared with cetuximab plus radiation ($P = 0.79$) or cisplatin plus radiation ($P = 0.54$). In the H520 xenografts, cetuximab alone and in combination with radiation, cisplatin or the triple combination did not significantly inhibit tumor growth.

with radiation in combination with radiation and chemotherapy. Studies are ongoing in our laboratory and other laboratories to define and validate tumor markers that will predict sensitivity or resistance to EGFR inhibitors. These studies are evaluating genes and proteins identified through gene expression profiling and proteomic analysis that are involved in EGFR signaling pathways as well as novel genes and proteins.

Table 6 Effects of cetuximab + fractionated radiation* and cisplatin on mean tumor volume in athymic mice bearing NCI-H292 tumors

Treatment	Tumor volume, day 93 (cm^3)	*P*
Cet vs. Control	4.2 ± 0.90 vs. 4.4 ± 2.5	0.80
RT vs. Control	2.2 ± 1.8 vs. 4.4 ± 2.5	0.049
Cet + RT vs. Control	1.5 ± 0.84 vs. 4.4 ± 2.5	0.012
CDDP + RT vs. Control	1.8 ± 1.4 vs. 4.4 ± 2.5	0.024
Cet + RT + CDDP vs. Control	1.2 ± 1.3 vs. 4.4 ± 2.5	0.004
Cet + RT + CDDP vs. Cet + RT	1.2 ± 1.3 vs. 1.5 ± 0.84	0.79
Cet + RT + CDDP vs. CDDP + RT	1.2 ± 1.3 vs. 1.8 ± 1.4	0.54

Abbreviations: Cet, cetuximab; RT, radiation; CDDP, cisplatin.
*5 Gy twice a week for 2 weeks.

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The Effects of Cetuximab Alone and in Combination With Radiation and/or Chemotherapy in Lung Cancer

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