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Dual-Agent Molecular Targeting of the Epidermal Growth Factor Receptor (EGFR): Combining Anti-EGFR Antibody with Tyrosine Kinase Inhibitor

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ABSTRACT

Molecular inhibition of epidermal growth factor receptor (EGFR/HER1) signaling is under active investigation as a promising cancer treatment strategy. We examined the potency of EGFR inhibition achieved by combining anti-EGFR monoclonal antibody and tyrosine kinase inhibitor, which target extracellular and intracellular domains of the receptor, respectively. We specifically studied the combination of cetuximab (Erbitux, C225; ImClone Systems, New York, NY) with either gefitinib (Iressa, ZD1839; AstraZeneca, Macclesfield, UK) or erlotinib (Tarceva, OSI-774; Genentech, South San Francisco, CA) across a variety of human cancer cells. The combination of cetuximab plus gefitinib or erlotinib enhanced growth inhibition over that observed with either agent alone. As measured by immunostaining, inhibition of EGFR phosphorylation with the combination of cetuximab plus gefitinib or erlotinib was augmented over that obtained with single-agent therapy in head and neck (H&N) cancer cell lines. Phosphorylation inhibition of downstream effector molecules [mitogen-activated protein kinase (MAPK) and AKT] also was enhanced in tumor cells treated with the combination of cetuximab plus gefitinib or erlotinib. Flow cytometry and immunoblot analysis demonstrated that treatment of H&N tumor cells with cetuximab in combination with either gefitinib or erlotinib amplified the induction of apoptosis. Following establishment of cetuximab-resistant cell lines, we observed that gefitinib or erlotinib retained the capacity to inhibit growth of lung and H&N tumor cells that were highly resistant to cetuximab. Treatment with gefitinib or erlotinib, but not cetuximab, also could further inhibit the activation of downstream effectors of EGFR signaling in cetuximab-resistant cells, including MAPK and AKT. These data suggest that tyrosine kinase inhibitors may further modulate intracellular signaling that is not fully blocked by extracellular anti-EGFR antibody treatment. Finally, animal studies confirmed that single EGFR inhibitor treatment resulted in partial and transient tumor regression in human lung cancer xenografts. In contrast, more profound tumor regression and regrowth delay were observed in mice treated with the combination of cetuximab and gefitinib or erlotinib. Immunohistochemical staining, which demonstrated significant reduction of the proliferative marker proliferating cell nuclear antigen in mice treated with dual EGFR inhibitors, further supported this *in vivo* observation. Together, these data suggest that combined treatment with distinct EGFR inhibitory agents can augment the potency of EGFR signaling inhibition. This approach suggests potential new strategies to maximize effective target inhibition, which may improve the therapeutic ratio for anti-EGFR-targeted therapies in developing clinical trials.

INTRODUCTION

The potential value of modulating epidermal growth factor receptor (EGFR) signaling as a cancer treatment approach is reflected by the broad array of molecular inhibitors that have been developed and launched in clinical trials during recent years. The timeline from the identification of epidermal growth factor in 1965 (1), to the postulation of EGFR signaling inhibition as an anticancer treatment strategy in 1983 (2), to the first Food and Drug Administration approval of an anti-EGFR agent in 2003 (3) is a reminder of the major challenge that faces each promising new agent seeking registration in cancer therapeutics. The majority of patients on clinical trials with anti-EGFR agents have been enrolled within the past 3–5 years, and several dozen large-scale trials remain in progress or in final design. Despite broad enthusiasm regarding the potential value of EGFR target modulation in cancer therapy, the field rests at an important crossroads in light of negative results from several large-scale phase III clinical trials in lung cancer reported in 2002–2003 (4, 5).

Negative clinical trial results invariably elicit "explanations" regarding outcome. Issues regarding lack of proper patient selection, inadequate drug dosing or scheduling, and suboptimal sequencing of EGFR inhibitor with cytotoxic therapy (and others) deserve systematic examination in preclinical and clinical settings. In this report, we explore the issue of maximizing EGFR target inhibition through the application of dual EGFR inhibitory agents of distinct molecular class. Specifically, the potential value of combining anti-EGFR monoclonal antibody (mAb) with EGFR tyrosine kinase inhibitor (TKI) to maximize EGFR signaling inhibition is examined using *in vitro* and *in vivo* model systems. The primary objective of this work is to examine whether combining distinct classes of EGFR inhibitors (mAb plus TKI) can augment the ultimate antitumor activity over that achievable with single EGFR inhibitor alone.

MATERIALS AND METHODS

Chemicals. Cell culture media were obtained from Life Technologies, Inc. (Rockville, MD). AstraZeneca (London, UK) provided gefitinib (ZD1839, Iressa). Genentech (South San Francisco, CA) provided erlotinib (OSI-774, Tarceva). ImClone Systems (New York, NY) provided cetuximab (C225, Erbitux). Primary antibodies against p-MAPK(Thr202/Tyr204) and p-AKT-(Ser473) and cleaved caspase-7 were obtained from Cell Signaling Technology (Beverly, MA). Antibody against proliferating cell nuclear antigen (PCNA) was obtained from Vector Laboratory, Inc. (Burlingame, CA). Anti-EGFR and anti-p-EGFR(Tyr1173) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-α-tubulin antibody was obtained from Oncogene Research Products (Cambridge, MA). All of the other chemicals were purchased from Sigma (St. Louis, MO).

Cell Lines. The human head and neck (H&N) squamous cell carcinoma (SCC) cell lines, UM-SCC1 (SCC-1) and UM-SCC6 (SCC-6), were provided by Dr. Thomas E. Carey (University of Michigan). Vulvar SCC (A431), prostate (PC3), and NSCLC (A549) cells were obtained from the American Type Culture Collection (Manassas, VA). Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas) provided the NCI-H226 (NSCLC) line. SCC cells were cultured routinely in DMEM supplemented with 10% fetal bovine serum, 1 μg/ml hydrocortisone, and 1% penicillin and streptomycin. Prostate and NSCLC cancer cell lines were maintained in complete culture media consisting of RPMI supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Human umbilical vascular endothelial cells were provided by Dr. Deane F. Mosher (University of Wisconsin-Madison) and cultured in MCDB 131-complete medium purchased from VEC Technologies, Inc. (Rensselaer, NY).

Establishment of Acquired Resistance to Cetuximab. During a 6-month period, tumor cells in culture were continuously exposed to increasing concentrations of cetuximab. Commencing with the IC_{50} of cetuximab for a particular tumor cell line, the exposure dose was progressively doubled every 10–14 days until 7–8 dose doublings had been successfully achieved. The

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established resistant cell lines then were maintained in continuous culture with the maximally achieved dose of cetuximab that still allowed cellular proliferation.

Growth Inhibition Assay. The antiproliferative effect of cetuximab in combination with either gefitinib or erlotinib for a variety of different cancer cell lines was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (6). Briefly, exponentially growing cancer cells were seeded into 96-well plates and incubated in medium containing vehicle control (DMSO), cetuximab alone, gefitinib or erlotinib alone, and cetuximab in combination with gefitinib or erlotinib for 48 h at 37°C. Duplicate plates containing six replicate wells per assay condition were seeded at a density of 1500 cells in 0.1 ml of medium. Following exposure of cells to anti-EGFR agents, 100 μl of MTT (1 mg/ml) were added to each well for 2 h at 37°C to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% SDS and 50% N,N-dimethylformamide. The absorbance of each well was measured in a microplate reader at 600 nm. The percentage cell growth was calculated by comparison of the A_{600} reading obtained from treated *versus* control cells.

Immunoblot Analysis. Following treatment, cells were lysed with Tween-20 lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin and aprotinin] and sonicated. Equal amounts of protein were analyzed by SDS-PAGE. Thereafter, proteins were transferred to nitrocellulose membranes and analyzed by specific primary antibodies against p-EGFR, EGFR, p-MAPK, p-AKT, and caspase-7. Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection system (Amersham, Piscataway, NY).

Assessment of Apoptosis. Apoptosis was detected by flow cytometry and immunoblot analysis via the examination of altered plasma membrane phospholipid packing and the activation of effector caspases in cells undergoing apoptosis. The membrane change was examined by the incorporation of the lipophilic dye MC540 in combination with DNA-specific dye Hoechst 33342 (Ho342) as described previously (7). Specifically, cells were harvested with 5 mM EDTA at 37°C. After centrifugation, cell pellets were resuspended in 900 μl PBS, followed by addition of 100 μl of 50 μg/ml Ho342. Thereafter, cells were incubated for 30 min in the dark, pelleted, and resuspended in 100 μl PBS. Four μl of MC540 (1 mg/ml) were added, and cells were incubated for 20 min in the dark. Cells were pelleted, resuspended in 1 ml PBS, and analyzed immediately by flow cytometry. MC540-positive cells were detected by an increase in red fluorescence, collected at 575 $\pm$ 20 nm, 0.5–2 log over 540-negative cells. Data were collected and analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). To further assess biochemical alteration in apoptotic cells, we examined expression of the active form of caspase-7, one of the downstream effectors in caspase signaling. Briefly, treated cells were harvested and processed for immunoblotting using primary antibody against cleaved capase-7 as described previously.

Assay of Tumor Growth in Athymic Nude Mice. Athymic nude mice (3–4-week-old females) were obtained from Harlan Bioproducts for Science (Indianapolis, IN) and maintained in a laminar air-flow cabinet under aseptic conditions. The care and treatment of experimental animals were in accordance with institutional guidelines. Human cancer cells ($\sim 1 \times 10^6$) were injected s.c. into the dorsal flank area of the mice on day 0. Tumor volume was determined by direct measurement with calipers and calculated by the formula: $\pi/6 \times$ (large diameter) $\times$ (small diameter)^2. Animal experiments generally included four treatment groups: vehicle control, cetuximab alone, gefitinib or erlotinib alone, and cetuximab in combination with gefitinib or erlotinib. Cetuximab was administrated by i.p. injection, and gefitinib or erlotinib was administered by oral gavage at the specified doses and intervals.

Immunohistochemical Determination of PCNA. The expression of PCNA (proliferative marker) was detected in histologic sections of tumor xenografts. Briefly, excised tumor specimens were fixed in 10% neutral buffered formalin. Following embedding in paraffin, 5-μm sections were cut, and tissue sections were mounted. Sections were dried, deparaffinized, and rehydrated. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, slides were incubated at 4°C overnight with 1:100 dilution of primary antibody directed against PCNA, followed by a 30-min incubation of secondary antibody. Slides then were incubated with streptavidin peroxidase and visualized using the 3,3′-diaminobenzidine chromogen (Lab Vision Corp, Fremont, CA).

Statistical Analysis. The effects of cetuximab and gefitinib/erlotinib on growth inhibition and apoptosis were assessed by multiple regression analysis using the PROC GLM procedure in SAS (version 8; SAS Institute, Inc., Cary, NC).

RESULTS

Effect of Cetuximab in Combination with Gefitinib or Erlotinib on Proliferation. We examined the antiproliferative effects of cetuximab in combination with gefitinib or erlotinib using the MTT assay in a variety of human tumor cell lines from H&N (SCC-1 and SCC-6), NSCLC (H226 and A549), or prostate (PC3), which express varying EGFR levels (Fig. 1*A*). MTT provides a measure of mitochondrial dehydrogenase activity within the cell and thereby offers an indication

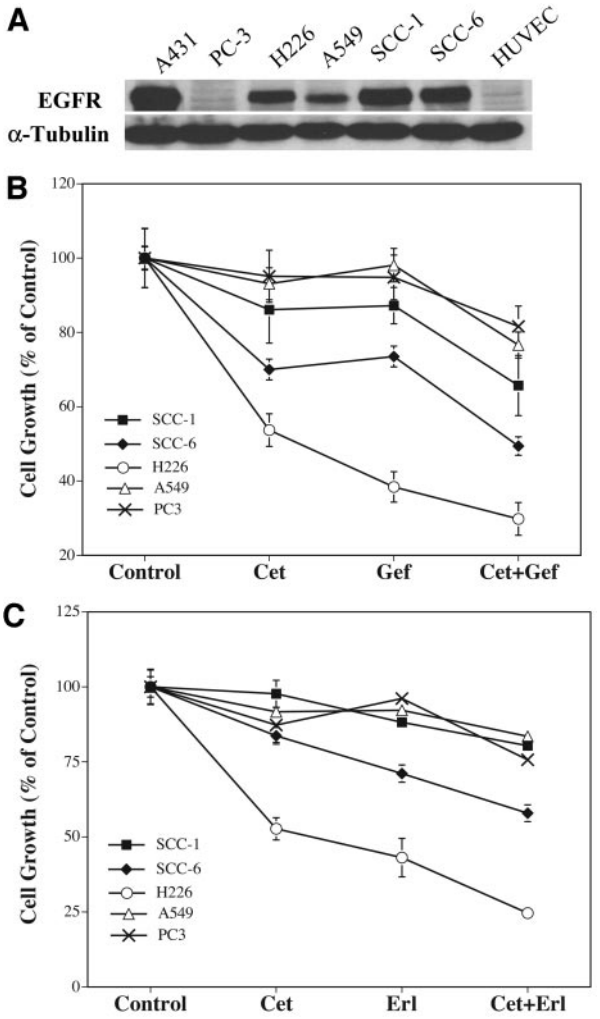

Fig. 1. Effect of cetuximab in combination with gefitinib or erlotinib on the proliferation of tumor cells. Antiproliferative effects of cetuximab in combination with gefitinib or erlotinib were examined in cell lines from head and neck (SCC-1 and SCC-6), NSCLC (H226 and A549), or prostate (PC3) cancer. *A*, cell lysates from a variety of cell lines were processed for immunoblot analysis using antibodies directed against epidermal growth factor receptor (*EGFR*) as described in "Materials and Methods." A431 cells and human umbilical vascular endothelial cells (*HUVECs*) serve as reference markers for high and low expression of EGFR. *B*, tumor cells (1500/well) were seeded into 96-well plates and subsequently exposed to cetuximab (5 nM) in combination with gefitinib (0.5 μM; *B*) or erlotinib (0.5 μM; *C*) for 48 h. The number of viable cells in each well was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in "Materials and Methods." Results were expressed as the percentage of cell growth relative to controls. Each point represents mean $\pm$ SD of five determinations. Similar results were obtained in replicate experiments. Data points are connected by lines for ease of data trend visualization, not because of linkage by chronology or dose.

of cellular proliferation status. As shown in Fig. 1*B*, using 5 nM and 0.5 μM of cetuximab or gefitinib, respectively, we observed modest growth inhibition in all of the five cell lines tested. Combining cetuximab with gefitinib resulted in more pronounced growth inhibition than single-agent treatment, particularly in the SCC-1 and H226 cells, which demonstrated greater than additive growth inhibition ($P < 0.05$). Among the cell lines tested, we did not observe clear correlation between the absolute level of EGFR expression and the degree of proliferative inhibition induced by exposure to dual EGFR inhibitory agents. Consistent with previous studies (8, 9), EGFR expression level alone is not likely the sole determinant influencing the antiproliferative activity of cetuximab or gefitinib. Similar results were observed in cells treated with cetuximab in combination with another EGFR TKI, erlotinib. Combined treatment with dual EGFR inhibitors resulted in enhanced growth inhibition compared with that achieved with single-agent exposure, particularly in the SCC-1, SCC-6, and H226 cells, which demonstrated greater than additive growth inhibition ($P < 0.05$; Fig. 1*C*). Together, these results suggest that treatment of tumor cells with dual anti-EGFR agents, which target distinct molecular domains of the EGFR, may augment one another in terms of antiproliferative activity.

Effect of Cetuximab in Combination with Gefitinib or Erlotinib on EGFR Signaling. To further characterize downstream EGFR signaling that might correlate with the observed growth inhibition, we examined the effect of anti-EGFR agents on the expression of several key regulators involved in the EGFR signaling pathway. As shown in Fig. 2, immunoblot analysis identified that single-agent treatment with EGFR inhibitors resulted in a reduction in phosphorylation of EGFR (p-EGFR) in SCC-1 cells. Treatment of cells with dual anti-EGFR agents further reduced the expression level of the activated form of EGFR. The level of activated mitogen-activated protein kinase (MAPK; p-MAPK), a downstream regulator of EGFR signaling, shows pronounced down-regulation in the face of dual anti-EGFR inhibitor treatment. Inhibition of p-AKT expression also was observed with dual anti-EGFR therapy, particularly with the combination of cetuximab and erlotinib. Similar results were confirmed in other cell lines, including H226 (data not shown). These results suggest that the inhibition of EGFR signaling achieved with the combination of cetuximab with gefitinib (or erlotinib) can be augmented beyond that achieved using each agent alone.

Effect of Cetuximab in Combination with Gefitinib on Apoptosis. We evaluated whether dual anti-EGFR inhibitors can augment cellular apoptotic response. Previous studies have demonstrated that EGFR inhibition, using either TKIs or anti-EGFR antibodies, can induce apoptosis in human tumor cells (10–12). We first examined apoptosis using MC540, which detects early stages of apoptosis (*i.e.*, conformational changes in the plasma membrane). As shown in Fig. 3*A*, cetuximab or gefitinib alone induced apoptosis to a similar degree (~2–5%) compared with control in SCC-1 and SCC-6 cells. Combined treatment with cetuximab and gefitinib resulted in more potent induction of apoptosis (increased threefold to fourfold compared with single agent), which confirmed a synergistic effect ($P < 0.03$). Similar results were observed when cells were treated with cetuximab and erlotinib as shown in Fig. 3*B*. The combined treatment with cetuximab and erlotinib showed additive impact in SCC-6 ($P = 0.078$) and synergistic impact in SCC-1 ($P = 0.02$) regarding apoptosis induction.

We further evaluated apoptotic response by examining levels of the active form of caspase-7. Caspases represent central regulators of apoptosis. Caspase signaling is initiated and propagated by proteolytic autocatalysis and cleavage of downstream caspases. Activation of effector caspases, such as caspase-7, leads to the cleavage of cytoskeletal and nuclear proteins such as lamin A and poly(ADP-ribose) polymerase, with resultant induction of apoptosis. As shown in Fig. 3*C*, using immunoblot analysis, we observed significant expression of active (cleaved) caspase-7 in both SCC cell lines treated with

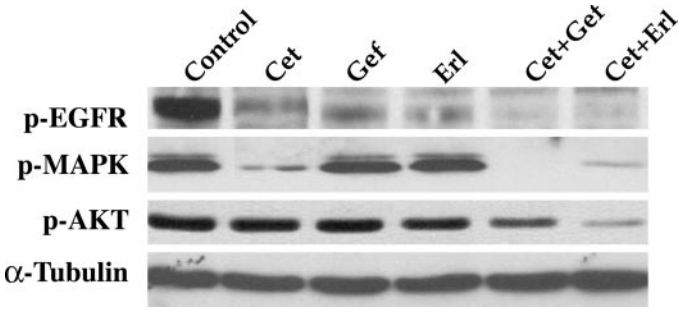

Fig. 2. Effect of cetuximab in combination with gefitinib or erlotinib on epidermal growth factor receptor (EGFR) downstream signaling. Exponentially growing SCC-1 cells were exposed to either single or dual anti-EGFR agents for 72 h. Following harvesting, cells were lysed and processed for immunoblot analysis using antibodies directed against p-EGFR, p-MAPK, and p-AKT as described in "Materials and Methods." The α-tubulin serves as a loading control.

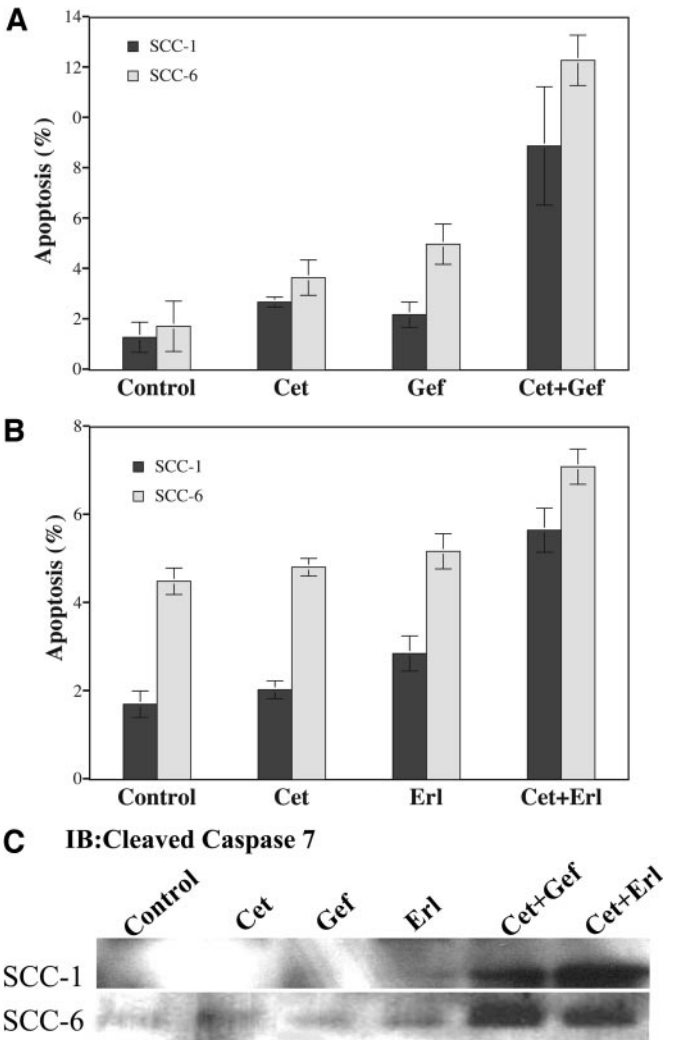

Fig. 3. Effect of cetuximab in combination with gefitinib or erlotinib on apoptosis of tumor cells. Apoptosis was examined by flow cytometry using MC540 (*A* and *B*) and immunoblot analysis using antibody against active form of caspase-7 (*C*) as described in "Materials and Methods." SCC-1 or SCC-6 cells were exposed to cetuximab (5 nM), gefitinib (0.5 μM), erlotinib (0.5 μM), or the combination for 72 h. The percentage of apoptotic cells was determined by quantifying the MC540-positive cell population. Data represent mean values from duplicate samples; *bars*, SD.

dual EGFR inhibitors but not in those cells treated with single-agent cetuximab, gefitinib, or erlotinib. This result further complements the flow cytometry data suggesting that dual anti-EGFR agents can augment cellular apoptotic response.

Effect of Gefitinib or Erlotinib on the Growth of Cetuximab-Resistant Tumor Cells. Although our results suggest that combining distinct classes of EGFR inhibitors can augment modulation of cellular proliferation, EGFR signaling, and apoptosis, limitations regarding the "optimal" (or clinically achievable) concentrations of each inhibitor remain. Therefore, we sought to evaluate whether the induction of resistance to one class of EGFR inhibitor would render cells cross-resistant to another class of EGFR inhibitor. To examine this hypothesis, we established cetuximab-resistant tumor cell lines through a process of stepwise dose-escalation exposure to cetuximab. Following ~6 months of progressive dose exposure, stably resistant SCC-6 and H226 cells were established that were capable of sustained growth in 640 nM cetuximab. As shown in Fig. 4*A*, these cetuximab-resistant SCC-6 and H226 cells maintained steady proliferation when exposed to increasing doses of cetuximab. In contrast, parental SCC-6 or H226 cells retained strong dose-dependent growth inhibition with cetuximab challenge. However, treatment with the small molecule EGFR TKIs gefitinib or erlotinib continued to induce clear growth inhibition of parental and cetuximab-resistant SCC-6 and H226 cells (Fig. 4, *B* and *C*). Among cells tested, we did not observe significant growth inhibition using the TKI solubilization vehicle (DMSO) alone (data not shown). These results indicate that EGFR TKIs retain their capacity to inhibit growth of tumor cells that have become highly resistant to anti-EGFR mAb. The data further suggest that TKIs can influence intracellular signaling that is only partially affected (or unaffected) by anti-EGFR mAb acting at the extracellular EGFR domain.

We further examined whether gefitinib or erlotinib could modulate EGFR signaling in cetuximab-resistant cells. To assay for activation of EGFR signaling, lysates from parental and cetuximab-resistant cells treated with anti-EGFR agents underwent immunoblot analysis with antibody specific for phosphorylated EGFR, MAPK, or AKT. As shown in Fig. 5, the expression of p-EGFR, p-MAPK and p-AKT was significantly reduced in parental cells treated with cetuximab (320 nM), gefitinib (6.4 μM), or erlotinib (6.4 μM). However, using identical dosing, EGFR downstream signaling remained essentially unaffected in cetuximab-resistant cells when challenged with cetuximab. These data complement the cetuximab-resistant phenotype observed in the proliferative growth profiles (Fig. 4*A*). Of particular interest, we found that gefitinib or erlotinib retained the capacity to inhibit downstream EGFR signaling in cetuximab-resistant cells as manifested by markedly decreased expression of p-MAPK and p-AKT. In parallel with the proliferation data (Fig. 4), this result indicates that despite clear establishment of cetuximab resistance, these cells are not cross-resistant to the TKIs gefitinib and erlotinib. These results suggest that, by directing inhibitors against distinct molecular domains of the EGFR, it may be possible to overcome certain limitations of a single EGFR inhibitor, including acquired resistance.

Effect of Cetuximab in Combination with Gefitinib or Erlotinib on Human Tumor Xenografts. We examined the effect of combining cetuximab with gefitinib or erlotinib on the growth of human tumor xenografts. H226 cells were inoculated s.c. into athymic mice and allowed to grow until they had achieved a mean volume of 100 mm^3 before treatments. To examine the *in vivo* interaction between cetuximab and gefitinib or erlotinib, doses of both agents were specifically selected so that their independent effects on tumor growth inhibition would be modest. Cetuximab was administered via i.p. injection at a dose of 0.2 mg twice per week for 4 consecutive weeks, and gefitinib was given by oral gavage at a dose of 0.5 mg, 5 days per week for 4 consecutive weeks. As shown in Fig. 6, treatment with cetuximab alone or gefitinib alone produced a modest growth inhibition in H226 xenografts. In contrast, combined treatment with cetuximab and gefitinib significantly inhibited tumor growth and resulted in substantial growth delay in the H226 xenografts. Similar results were observed in mice treated with cetuximab in combination with erlotinib (Fig. 6). Using the same control and cetuximab-treated mice as a reference, the combination of cetuximab and erlotinib resulted in significant inhibition of tumor growth and growth delay in the H226 tumor xenografts.

The expression of molecular markers of tumor proliferation was further examined in histopathologic sections taken from H226 tumor xenografts. Immunohistochemical staining with PCNA demonstrated the number of proliferating cells to be greatest in the control group; intermediate in the groups receiving single-modality treatment with cetuximab, gefitinib, or erlotinib; and least in the group receiving dual EGFR inhibitor treatment (Fig. 7). These results complement previous data and suggest that combining distinct classes of EGFR inhibitors may augment one another via inhibition of cellular proliferation.

DISCUSSION

Promising characteristics of the EGFR as a molecular target for cancer therapy have prompted an extensive drug development effort to design pharmacologic inhibitors of EGFR signaling. The agents developed can be crudely classified into two major categories, namely, large molecule anti-EGFR antibodies and small molecule EGFR TKIs. In early clinical trials, these inhibitors have been primarily evaluated in either the monotherapy setting or in combination with conventional cytotoxic agents. Limited data have been generated regarding the capacity of multiple EGFR inhibitors to work in concert with one another.

In the current study, we present data from *in vitro* and *in vivo* studies to suggest that combining distinct classes of EGFR inhibitors may augment ultimate antitumor activity. By combining agents that target distinct molecular domains of the EGFR (*e.g.*, cetuximab targets the extracellular ligand-binding domain, whereas gefitinib and erlotinib target the intracellular receptor kinase domain), the results suggest that a complementary impact on downstream signaling, apoptosis, proliferation, and tumor xenograft growth can be achieved. Furthermore, following establishment of cetuximab-resistant cell lines, we confirm that gefitinib and erlotinib retain the capacity to inhibit cellular growth and downstream EGFR signaling in the cetuximab-resistant cells (Fig. 4 and Fig. 5). These results suggest that combining distinct classes of EGFR inhibitors may not only potentiate cellular toxicity caused by nonoverlapping mechanisms of action but also may assist to overcome inherent or acquired resistance to one class of EGFR inhibitor.

There are several potential explanations regarding how/why combining distinct EGFR inhibitors may augment antitumor effectiveness over that achieved with single-agent therapy. First, there are rare examples of pharmacologic inhibitors that successfully exert 100% influence over their respective target. By directing inhibitors against distinct molecular domains of the EGFR, we may overcome inherent limitations of any single inhibitor. Second, the EGFR downstream signaling pathway is anything but linear (13). Specific poly(ADP-ribose) polymerase receptor dimerization profiles (particular homodimer or heterodimer combinations) can influence the intensity and direction of predominant downstream signaling and resultant cellular effects. Certain ErbB dimers appear to favor signaling along the RAS/RAF/MAPK pathway, some along the phosphatidylinositol 3′-kinase/AKT pathway, and still others along signal transducer and activator of transcription (STAT) pathways. Third, EGFR inhibitors

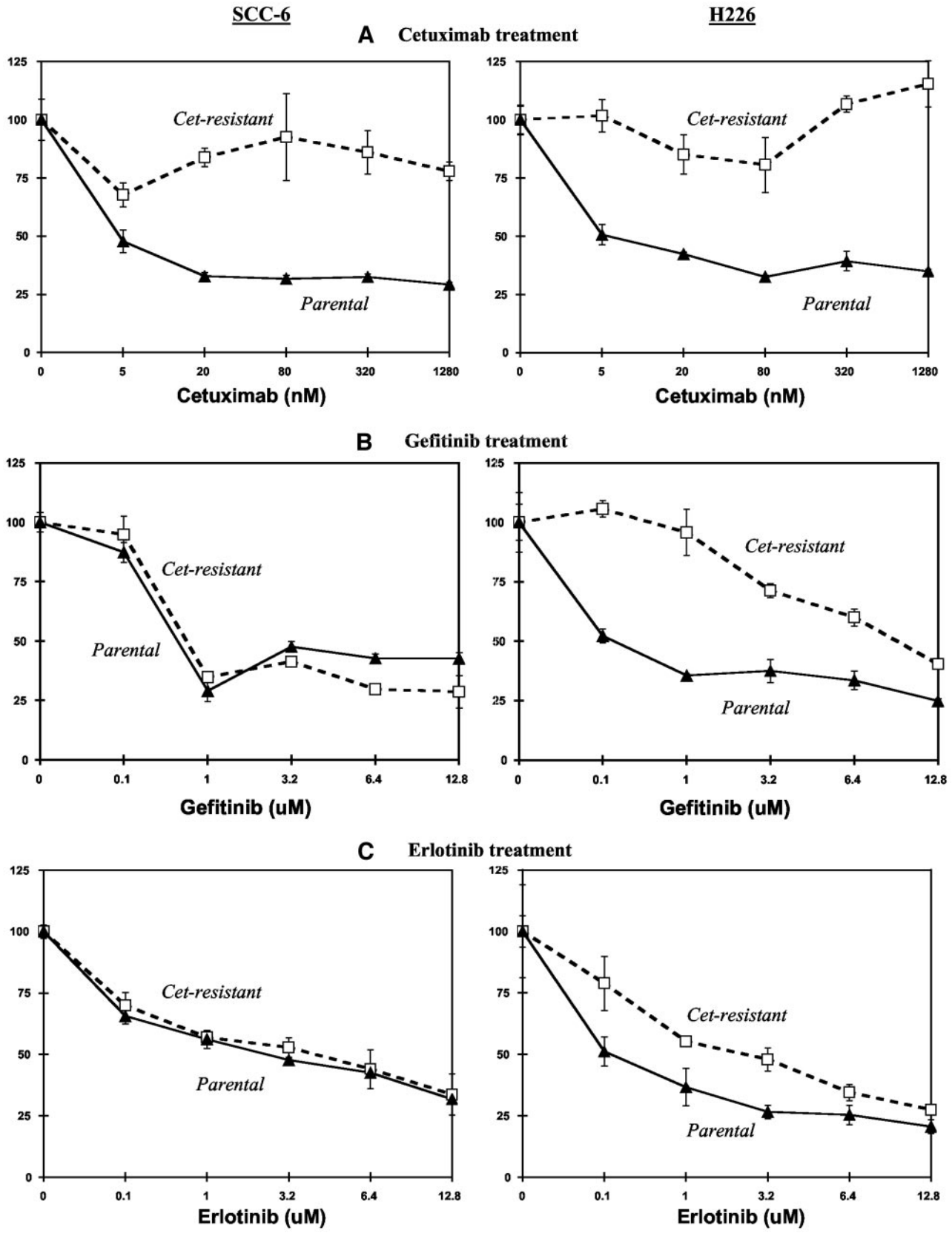

Fig. 4. Effect of gefitinib or erlotinib on the proliferation of cetuximab-resistant cells. Cetuximab-resistant SCC-6 or H226 cells were established as described in "Materials and Methods." Parental or cetuximab-resistant tumor cells (1500/well) were seeded into 96-well plates. Cells subsequently were exposed to indicated doses of cetuximab, gefitinib, or erlotinib for 4 days. The number of viable cells in each well was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in "Materials and Methods." Results were expressed as the percentage of cell growth relative to controls.

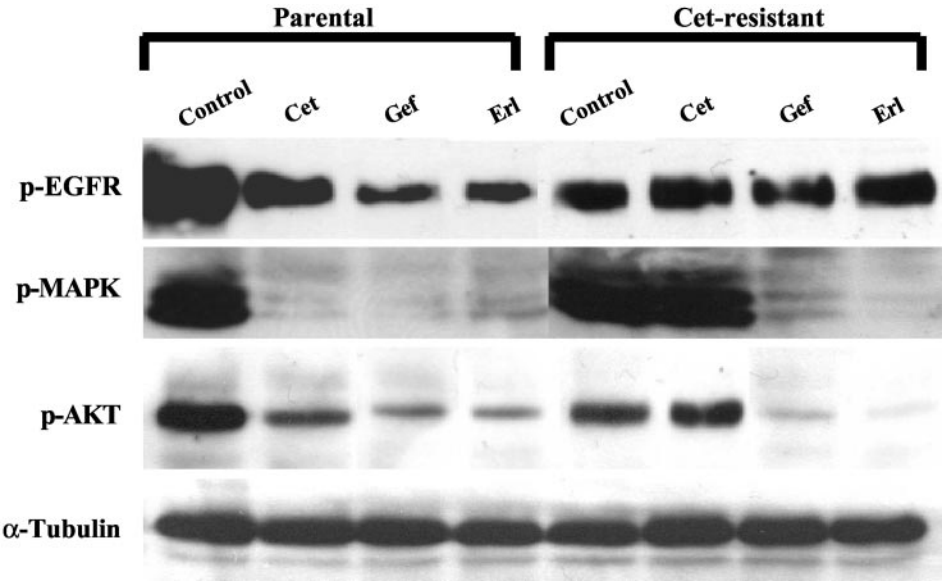

Fig. 5. Effect of gefitinib or erlotinib on the epidermal growth factor receptor (EGFR) signaling of cetuximab-resistant cells. Cetuximab-resistant H226 (C640) cells were established as described in "Materials and Methods." Parental or cetuximab-resistant H226 tumor cells were exposed to cetuximab (320 nM), gefitinib (6.4 μM), or erlotinib (6.4 μM) for 4 days. Following harvesting, cells were lysed and processed for immunoblot analysis using antibodies directed against p-EGFR, p-MAPK, and p-AKT as described in "Materials and Methods." The α-tubulin serves as a loading control.

are not absolute in their specificity for the EGFR tyrosine kinase. EGFR TKIs in particular show varying degrees of cross-reactivity for a spectrum of receptor tyrosine kinases, which may play a role in their capacity to augment resultant cellular effects when combined with the more selective anti-EGFR mAb (14, 15). This also may explain why TKIs can further inhibit the growth of tumor cells that have acquired resistance to anti-EGFR mAbs. This "imperfect" selectivity of TKIs for EGFR may play a favorable role by allowing partial inhibition of adjacent ErbB or other tyrosine kinases that can modulate downstream molecules of EGFR signaling and cellular proliferation. This could explain the observation of significant inhibition of p-MAPK and p-AKT but not p-EGFR in cetuximab-resistant cells when treated with gefitinib or erlotinib (Fig. 5).

Consistent with speculation described previously, several recent reports suggest that dual-agent targeting of the EGFR or ErbB family of receptors warrants additional investigation (16–18). Matar *et al.* (16) studied the A431 cell line and showed that the combination of cetuximab and gefitinib could augment inhibitory effects on cell proliferation and tumor xenograft growth. Dowlati *et al.* (17) examined the combination of the small molecule EGFR inhibitor AG1478 with the Janus-activated kinase/STAT inhibitor AG490 (inhibits STAT-3 phosphorylation) and found complementary growth inhibition in A431 cells. Several groups have investigated antitumor activity with combined EGFR and ErbB-2 inhibitors and observed potentiation in some (19, 20) but not all models (21). Several of the EGFR inhibitors being developed are themselves dual-ErbB or pan-ErbB inhibitors, which will offer the opportunity for outcome comparisons with single-ErbB inhibitors.

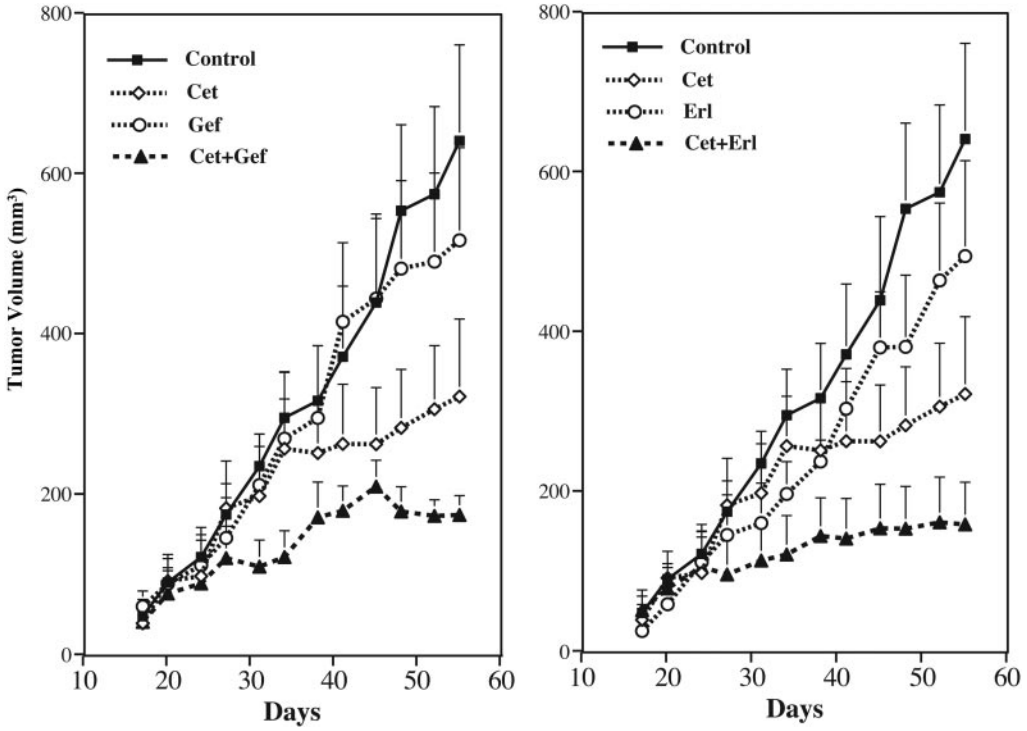

Fig. 6. Antitumor activity of cetuximab in combination with gefitinib or erlotinib in tumor xenografts. H226 (10^6) cells were injected s.c. into the dorsal flank of athymic mice as described in "Materials and Methods." After 20 days (tumor mean size, 100 mm^3), treatment was initiated by injecting 0.2 mg of cetuximab i.p. twice per week for a total of eight injections. Gefitinib (0.5 mg) or erlotinib (0.2 mg) was delivered via oral gavage 5 days per week for 4 consecutive weeks. Control mice received human IgG as antibody control and Tween-80 solution as vehicle control. Values represent mean tumor size ($n = 8$ per group).

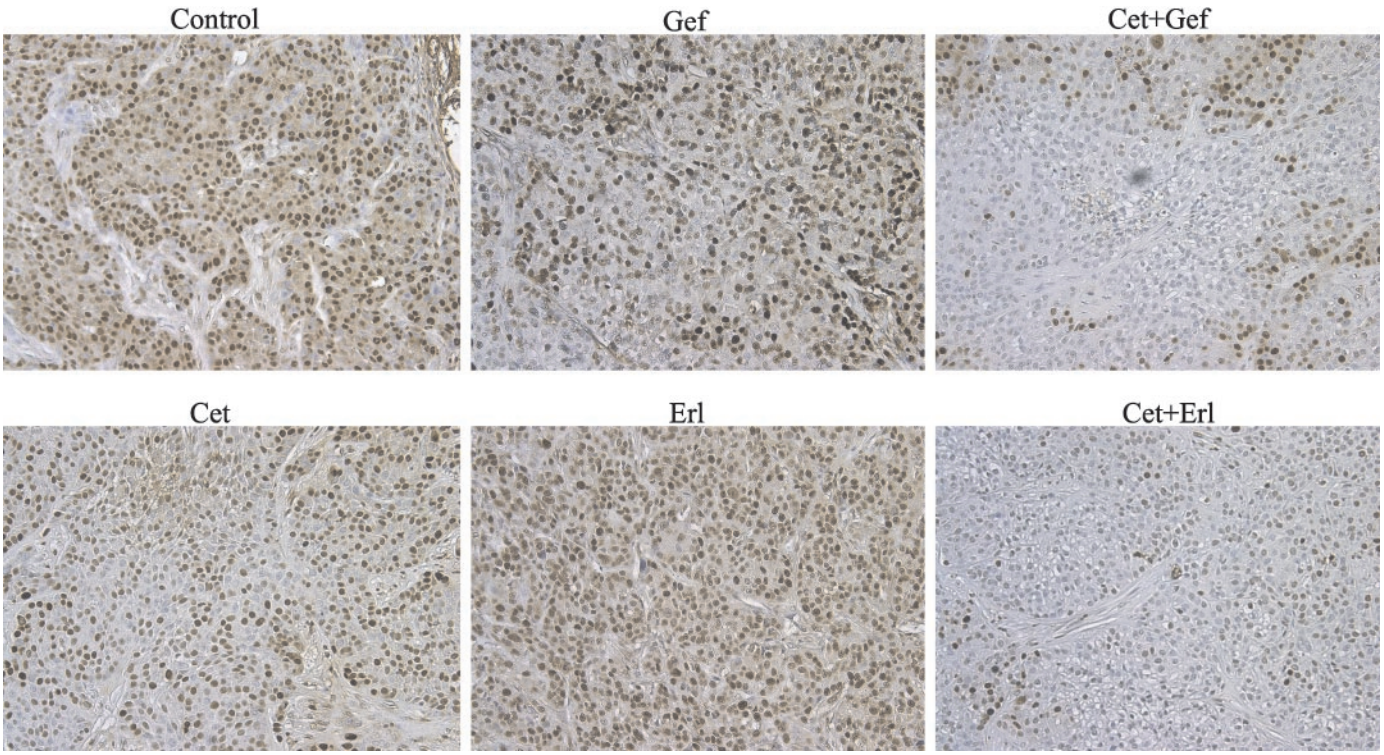

Fig. 7. Immunohistochemical analysis of cellular proliferation [proliferating cell nuclear antigen (PCNA)]. Immunohistochemical staining for PCNA was performed using representative human H226 tumor tissue sections taken from mice treated with vehicle control or single or dual anti-EGFR agents at day 56 following tumor inoculation. Positive (*brown*) staining indicates expression of PCNA.

Reliable methods to predict those patients who are most likely to respond to EGFR inhibitor therapies remain unknown at this time. In contrast to experience with ErbB-2 overexpression in breast cancer patients, which predicts response to trastuzumab, the logical supposition that tumors overexpressing EGFR would respond best to EGFR inhibitory strategies has not been borne out by clinical trials (22, 23). More extensive evaluation of EGFR pathway and related markers, including p-EGFR, p-MAPK, p-AKT, STAT-3, p27, Ki67, and others, may help to establish predictive "molecular fingerprints" of those tumors most likely to respond favorably to EGFR inhibition (24–28). In support of this hypothesis, we found that baseline levels of p-EGFR, p-MAPK, and p-AKT in the cetuximab-resistant cells were low and remained essentially unaffected when challenged with cetuximab (Fig. 5). In contrast, baseline levels of p-EGFR, p-MAPK, and p-AKT in the parental cells were high and were significantly reduced following cetuximab challenge. This result suggests that the capacity of tumor cells to maintain active signaling through phosphatidylinositol 3′-kinase/AKT and MAPK pathways may represent a potential mechanism of resistance by which tumor cells escape the antiproliferative impact of EGFR inhibitors.

In parallel to the current results, several recent reports suggest that constitutively active MAPK and AKT may contribute to resistance to EGFR inhibitors (29–33). Investigating a panel of tumor cell lines, Janmaat *et al.* (30) found that EGFR inhibitors induced growth inhibition in A431 but not in a series of lung cancer cell lines. Further, persistent activity of either MAPK or phosphatidylinositol 3′-kinase/AKT pathways was observed in the resistant lung cancer lines (30). Using MDA-468 breast cancer cells, Bianco *et al.* and She *et al.* (32, 33) showed that resistance to gefitinib was associated with loss of PTEN and consequent hyperactivation of AKT with uncoupling of the AKT pathway from EGFR. Reconstitution of PTEN in these cells re-established EGFR-driven AKT signaling and thereby restored gefitinib sensitivity. These results suggest that MAPK and/or AKT signaling pathways may play a central role in the development of resistance to EGFR inhibitors. We are actively examining molecular characteristics of our acquired EGFR inhibitor-resistant cell lines in an effort to clarify mechanisms contributing to this resistance. Identification of potential predictive factors of response, or resistance, to EGFR inhibitors may provide valuable clinical insights, which could allow for more precise selection of specific therapeutic strategies for cancer patients.

Many human solid tumors acquire considerable biologic and genetic heterogeneity as they evolve. Although some tumors may rely heavily on EGFR signaling for their growth advantage, it is likely that several distinct molecular signaling pathways contribute to unchecked progression. If true, specific targeting of distinct molecular pathways may be required to effect a meaningful overall clinical response. This theme underlies recent efforts to combine molecular inhibitors from distinct classes in an attempt to combat tumor heterogeneity. Nonetheless, even our capacity to "maximally" inhibit a single molecular target such as the EGFR with current single-agent approaches would appear suboptimal. The data presented herein suggest that combining distinct classes of EGFR inhibitors can augment the antitumor response over that realized with a single EGFR inhibitor. The data further suggest that acquired resistance to one class of EGFR inhibitor may be partially overcome by challenge with another class of EGFR inhibitor. These preclinical data from the *in vitro* and *in vivo* setting warrant validation across other laboratories and may provide a scientific platform for the future design of clinical trials, which further explore this dual EGFR inhibitor strategy.

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