FINAL REPORT
J.W. Fulbright Association of Spanish Fulbright Alumni Grant
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Receiving this grant has represented an honor, a responsibility, but also an opportunity to continue a research carrer in the development of new treatment options for cancer patients. The focus of our group is to describe the efficacy, but also to gain insight into the mechanism of action and resistance of targeted drugs. The lines of investigation of the group are various, but my personal contribution during my first year in the group has been to investigate the mechanism of action but especially of resistance to epidermal growth factor receptor (EGFR) agents.

SUMMARY OF RESEARCH

Analysis of gene expression of cancer cell lines exposed to erlotinib, a small molecule inhibitor of the EGFR, showed a marked increase in EGFR mRNA in resistant cell lines but not in susceptible ones. Because cetuximab induces EGFR downregulation, we explored the hypothesis that treatment with cetuximab would interfere with erlotinib-induced EGFR upregulation and result in antitumor effects. Exposure of the resistant biliary tract cancer cell line HuCCT1 but not the susceptible A431 epidermoid cell line to erlotinib induced EGFR mRNA and protein expression. Combined treatment with cetuximab blunted the erlotinib-induced EGFR upregulation and resulted in inhibition of cell proliferation and apoptosis in the HuCCT1 cells. Blockage of erlotinib-induced EGFR synthesis in HuCCT1 cells by siRNA resulted in identical antitumor effects as cetuximab providing mechanistic specificity. In mice xenografted with HuCCT1, A431, and the pancreatic cancer cell line Panc430, maximal growth arrest and decrease in Ki67 proliferation index was documented with combined therapy, and EGFR downregulation was observed in cetuximab-treated tumors. These results may indicate that resistance to EGFR kinase inhibition may be, at least in part, mediated by a highly dynamic feed-back loop consisting of upregulation of the EGFR upon exposure to EGFR kinase inhibitors. Abrogation of this response by siRNA-mediated EGFR mRNA downregulation and/or by cetuximab-mediated protein clearance induced tumor arrest across several cancer models with different EGFR expression levels, suggesting that resistance and sensitivity are dynamic events were proportional decrease in the target rather than absolute content dictates outcome.

RESEARCH ACCOMPLISHED

The epidermal growth factor receptor (EGFR) is a membrane receptor with an extracellular domain, a single alpha-helix transmembrane domain, and an intracellular domain with tyrosine kinase (TK) activity. EGFR signaling increases proliferation, angiogenesis, metastasis, and decreases apoptosis. EGFR expression is associated with a worse prognosis in a wide array of malignancies, and it constitutes a therapeutic target in active development. Two major strategies have been developed to target the EGFR: the use of small molecules that compete with adenosine triphosphate for binding to the kinase pocket, and the use of monoclonal antibodies (MAbs) directed against the external domain of the receptor. EGFR-directed therapies have shown a consistent but low level of clinical activity across tumor types and factors determining their efficacy are largely unknown. In addition, little is known about the effect of EGFR-targeted agents at the molecular level, the response that these agents elicit in the cell machinery, and whether this response may be relevant in spontaneous and acquired resistance. This study was

conducted to determine the mechanisms of resistance to TK inhibitors, and to devise rational ways of targeting the EGFR as an anticancer therapy.

An exploratory, broad-range evaluation was performed assessing the changes in gene expression occurring after treatment with erlotinib, a quinazoline derivative that reversibly inhibits the TK of EGFR, shows *in vitro* and *in vivo* activity in human cancer cell lines (1, 2), and has recently received approval as second or third line therapy in patients with non-small cell lung cancer (NSCLC). After treating naturally resistant (HuCCT1, IC_{50} > 20 μM) and naturally sensitive (SNU308, IC_{50} < 1 μM) biliary cancer cell lines with erlotinib, 18 and 12 genes were upregulated after 1 hour of treatment (Table 1), and 41 and 61 after 24 hours, respectively. Among them, a significant increase in EGFR mRNA levels was observed in HuCCT1 cells compared to baseline at 1 and 24 hours ($p = 0.028$ and $p = 0.017$, respectively), whereas no change in EGFR mRNA was documented in SNU308 cells. Considering this differential reaction in terms of gene expression of a target in response to pharmacological inhibition in resistant versus sensitive cell lines and its potential mechanistic implications, we conducted further experiments to better understand the molecular and translational implications of these findings.

We then investigated the pattern of EGFR mRNA response to EGFR-targeted therapies using RT-PCR and ELISA analysis to evaluate EGFR mRNA and protein dynamics, respectively. Because cetuximab (a quimeric mouse-human MAb that induces downregulation of the EGFR)(3) induces EGFR downregulation, we explored the hypothesis that it would interfere with erlotinib-induced EGFR upregulation. Treatment of HuCCT1 cells confirmed that erlotinib induced EGFR mRNA synthesis (Fig. 1A). Furthermore, this upregulation of mRNA levels was closely followed by a 2-fold increase in the transcription of the protein (Fig. 1B). A decrease in protein was observed both in cells treated with cetuximab alone and with the combination, suggesting that cetuximab is able to abrogate the erlotinib-induced increase in EGFR transcription. In EGFR-dependent, erlotinib-sensitive (IC_{50} < 1 μM) A431 squamous carcinoma cells there was no differential increase in EGFR mRNA after treatment with erlotinib (Fig. 1C). Erlotinib did not induce an increase of EGFR, and cetuximab induced less significant changes in protein content compared to control cells than in HuCCT1 cells (Fig. 1D).

To examine whether the abovementioned differences were related to differential EGFR signaling, we conducted western blot analysis of both cell lines in the presence of the two drugs alone and in combination at different time points (Fig. 1E). These studies documented a significant (and identical) inhibition of EGFR activation in both cell lines by erlotinib, cetuximab, but especially with the combination. Inhibition of MAPK was more profound in A431; in HuCCT1 Akt activation was not significantly inhibited by any agent alone, and the effect of the combination was discrete.

In order to fully assess the hypothesis that resistance to erlotinib in HuCCT1 cells may be in part mediated by the erlotinib-induced upregulation of the target, we aimed at downregulating EGFR transcription by means of interfering with EGFR mRNA content. HuCCT1 cells were transfected with small interference RNA (siRNA) against the EGFR, and treated during 24 hours with erlotinib, cetuximab, and the combination (Fig. 2). EGFR mRNA synthesis decreased in control cells by 50%, and was not affected by treatment with the transfection reagent alone (data not shown). The induction of EGFR mRNA by erlotinib was efficiently abrogated by EGFR siRNA.

We confirmed that EGFR protein levels closely followed mRNA dynamics, as evidenced by the decrease in protein levels induced by EGFR siRNA; cetuximab and EGFR siRNA showed an additive effect in diminishing EGFR. The combination induced a non-significantly higher growth arrest compared with no treatment or single agent erlotinib/cetuximab (data not shown), and siRNA treatment enhanced this effect. In non-transfected cells the combined treatment induced a 5.6 to 7.4-fold increase in cell apoptosis when compared with erlotinib or cetuximab treatment alone, respectively. In siRNA-transfected cells apoptosis was higher in all treatment modalities compared with no transfection, but this difference was considerably superior in erlotinib-treated cells.

To confirm the molecular events described before, and to determine the effect of these drugs in a model closer to a clinical context, we tested our hypothesis in several *in vivo* models. Cancer cell lines with high (A431, vulvar), intermediate (HuCCT1, biliary), and low (Panc430, pancreatic) EGFR expression levels were xenografted in mice that were subsequently treated (Fig. 3). In A431-bearing mice erlotinib induced short-lasting growth arrest, cetuximab prompted a more delayed tumor control, and the combination seemed to achieve both a rapid and a durable tumor growth arrest. Cetuximab significantly decreased EGFR levels by day 1 of treatment. In HuCCT1-bearing mice cetuximab was equally effective in controlling growth when given alone or in combination with erlotinib, and EGFR levels equally decreased in both groups. To test whether cetuximab efficacy was dependent on prior EGFR-directed therapy, and to further assess the hypothesis that growth correlated with EGFR content, mice initially allocated to erlotinib received additional cetuximab (4 doses in 14 days) after completion of erlotinib therapy, a growth interruption was observed, and EGFR levels decreased. In Panc430 xenografts the combined treatment started to show a marginally higher effect than the individual drugs after 28 days. EGFR consistently decreased in these tumors after treatment with cetuximab, although only in the combined therapy arm this decrement was accompanied by growth arrest. In an attempt to correlate *in vivo* efficacy with immunohistochemical efficacy endpoints, we evaluated the variations in the proliferative index Ki67 in paraffin specimens of HuCCT1 and A431 tumors after 14 days of treatment (Fig. 3D). A significant decrease in Ki67 staining (from 3+ to 2+) was documented in HuCCT1 tumors treated with cetuximab, but especially in those treated with erlotinib and cetuximab (from 3+ to 1+), whereas no significant variation in proliferation was observed in erlotinib treated tumors. In A431, where a baseline higher proliferation was documented (4+), erlotinib and cetuximab decreased Ki67 staining to 2+, whereas the combined treatment resulted in a 1+ score.

The observation that a cell line responded to the inhibition of a given enzymatic activity initiating a compensatory feed-back loop that in a matter of hours incremented the total amount of this enzymatic activity is a rather classic pharmacologic paradigm, representing a homeostatic, adaptative mechanism to overcome target inhibition. Therefore, resistance and sensitivity may be redefined as the ability or inability of the cell to adapt to a changing environment, and the fact that decreasing the target while maintaining a constant drug concentration dramatically increases cell kill as assessed by apoptotic indexes suggests that the ultimate outcome is dictated by dynamic processes and quantitative ratios of drug/target rather than static, qualitative features. This compensatory effect may explain some apparently paradoxical findings observed in several clinical trials (4), where upregulation of phosphorylated EGFR was observed after treatment with erlotinib in breast cancer patients. In another report, modifications of EGFR serum values

during treatment for NSCLC seemed to reflect gefitinib activity; responding patients had decreasing EGFR serum levels compared with refractory patients, where an increment from baseline was observed (5). In our model, physically decreasing the amount of protein using an extracellular-acting monoclonal antibody increased sensitivity to the inhibition of the kinase activity by an intracellular-acting small molecule, inducing a synergistic effect in terms of induction of apoptosis *in vitro*, and an additive effect in terms of tumor growth arrest *in vivo*. The abrogation of the compensatory feed-back loop with siRNA rendered the cell defenseless to the pharmacologic insult, reverting an innate resistance to erlotinib. However, at present we are unable to define the cellular mechanism that senses and transduces EGFR functional status, and this leads us to the second implication of this report, namely the potential of dual targeting strategies.

Recent reports have shown additive effect of a TK inhibitor in combination with cetuximab in head and neck cancer (6) and A431 (7) models. The mechanism responsible for this higher efficacy of dual targeting was not addressed in those reports, and to our knowledge this is the first insight into a potential mechanistic explanation of that observation. We can hypothesize that the cell may avoid entering apoptosis either with part of the receptor pharmacologically inhibited, or with less total amount of receptor, but is unable to cope with the impact of both modulations. This was confirmed with a proof-of-principle strategy (short-circuiting the upregulating response with siRNA) that showed that the cell could cope with one but not two inhibitory apporaches. This threshold effect may be supported by recent reports suggesting that the presence of certain mutations in the catalytic domain of the EGFR augment the sensitivity of cells and tumors to gefitinib (8, 9). One of this reports demonstrates that transfection of the mutated receptor to a naturally resistant, EGFR wild-type cell line induces sensitivity to a constant gefitinib concentration (8), indicating that these mutations may turn the receptor susceptible to a clinically achievable drug concentration range, that is in turn unable to efficaciously inhibit the wild-type receptor in the majority of the patients. It is relevant to note that in consonance with prior reports (10), siRNA-mediated EGFR downregulation by itself had no effect on cell growth and/or apoptosis, and that the factor implicated in maximal apoptosis was erlotinib treatment. In contrast, other reports show that siRNA of the EGFR in A549 lung cancer cells by downregulating the receptor inhibited cellular proliferation and motility, and enhanced chemosensitivity to cisplatin (11). An even more provocative report documented that intravenous siRNA therapy targeting the EGFR prolonged survival in a glioma model (12).

The third significant aspect of this report is the observation that proportional decreases in EGFR rather than absolute baseline protein content dictated growth arrest. Both monoclonal antibody- or siRNA-mediated targeting of the EGFR provided evidence of a positive correlation between EGFR proportional protein content and growth, regardless of the method of evaluation used (*in vitro* cell viability, *in vitro* apoptosis, *in vivo* tumor growth, or *in vivo* proliferation assessment by Ki67). It is noteworthy that Panc430 tumors presented EGFR levels 17- and 12-fold lower than A431 and HuCCT1 tumors, respectively. Notwithstanding, cetuximab decreased EGFR in the same proportion (40% to 50% from baseline after 14 days of treatment), and achieved a growth inhibitory effect that was significant across all three models. Activity of gefitinib has been observed in cells that express high and low levels of EGFR (13), and synergistic effects along with chemotherapeutic agents was not dependent upon a high level of EGFR expression (14). The former observations have been documented also in a clinical setting: EGFR status correlates

poorly with response to both monoclonal antibodies (15, 16) and TK inhibitors (17, 18). The present findings indicate that EGFR regulation is highly sensitive and dynamic, significant changes can occur in short periods of time, and EGFR-directed therapy itself may induce such changes. Strategies consisting in seriated biopsies may be preferable to single, baseline evaluation to accurately evaluate EGFR dynamics in a clinical setting.

PRACTICAL APPLICATION AND IMPLICATIONS OF THIS RESEARCH

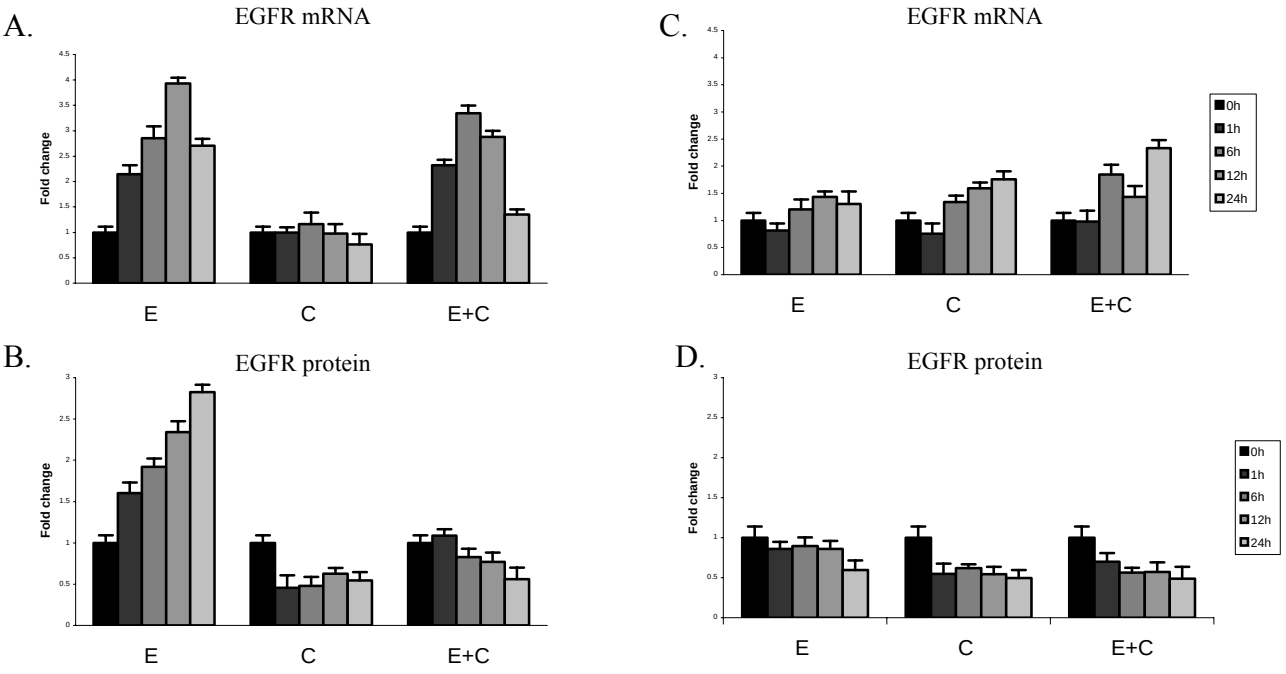
The success observed by our group (and others) in the combination of two different strategies targeting the EGFR has prompted an effort to translate this into the clinic. Under the leadership of the Director of the Drug Development and Gastrointestinal Cancer Programs, Manuel Hidalgo M.D., Ph.D., we are conducting a Phase I clinical trial in patients with solid tumors assessing the combination of erlotinib and cetuximab. This clinical study incorporates translational research studies (multiple tumor biopsies) to evaluate this paradigm. The central hypothesis of the study is that treatment with EGFR-targeted therapies induces variations in EGFR content, and the pattern of this variation is predictive of the efficacy of the combination. The proposed studies are structured around a phase I protocol (written by myself as the Clinical Fellow of the Drug Development Program) that is being developed at our Institution combining erlotinib with cetuximab in patients with solid tumors amenable to sequential biopsies. The study includes a "lead-in" period of either agent for two weeks before starting the combined modality portion of the study. This design permits the collection of normal and tumor tissues under treatment with both erlotinib and cetuximab alone to dissect the pharmacological effects of both drugs. The proposed multidisciplinary study represents a unique opportunity to address major gaps of knowledge concerning the dynamic behavior of the EGFR in response to therapy, and its value as a predictor of efficacy to EGFR-targeted therapy.

APPENDIX A: TABLES AND FIGURES

Table 1.
Genes with significant (P value < 0.05) upregulation or downregulation after 1 hour of treatment with erlotinib in HuCCT1 and SNU308 cells. Microarray hybridization was performed on the Affimetrix U133A gene array, containing ~22,000 unique human transcripts. Sample preparation and processing were performed as described in the Affimetrix GeneChip® Expression Analysis Manual (Affimetrix Inc., Santa Clara, CA). The CEL files generated by the Affimetrix Microarray Suite (MAS) version 5.0 were converted into DCP files using dCHIP (www.dCHIP.org), as described previously (19). Genes that were differentially expressed 3-fold or greater in 0 versus 1 or 0 versus 24 hours were then identified by defining the appropriate filtering criteria in the dCHIP software (mean E/ mean B>3; mean E-mean B=100; p<0.05, t-test).

	Gene	Fold	P value
HuCCT1	Hypothetical protein FLJ20047	24.3	0.040
	Consensus 217P22 on 6p21.1-21.31/Dynein heavy	23.8	0.008
	Epidermal growth factor receptor	18.4	0.028
	Consensus AF052090.1/23950 mRNA /mRNA	13.7	0.028
	Consensus M78162 /gi:273899 /EST01755	9.4	0.012
	Chymotrypsinogen B1	9.2	0.047
	Lipoprotein, Lp(a)	7.1	0.014
	Interleukin 6 (interferon, beta 2)	4.3	0.030
	Down syndrome critical region gene 1	3.7	0.032
	Interleukin 11	3.7	0.026
	Transcription factor 8 (represses interleukin 2 expression)	3.3	0.035
	Gastric intrinsic factor (vitamin B synthesis)	3.1	0.044
	Tenascin XB	-3.2	0.025
	Polymerase (RNA) II (DNA directed) polypeptide B (140kD)	-3.6	0.048
	Parvin, beta	-4.0	0.039
	Bone morphogenetic protein 2	-4.1	0.023
	Glycoprotein M6B	-6.9	0.036
	Chimerin (chimaerin) 2	-7.0	0.039
SNU308	Connective tissue growth factor	13.9	0.019
	v-jun sarcoma virus 17 oncogene homolog	12.9	0.032
	Cysteine-rich, angiogenic inducer, 61	12.9	0.021
	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	6.8	0.019
	Hypothetical protein FLJ20972	5.4	0.044
	Prostaglandin E receptor 4 (subtype EP4)	5.2	0.045
	Activating transcription factor 3	4.6	0.029
	Tenascin XB	3.7	0.027
	Hypothetical protein FLJ20071	-3.0	0.020
	Consensus AK025247.1/FLJ21594/10437718/Hs.288571/FLJ21594	-4.6	0.041
	Solute carrier family 12 (sodium/potassium/chloride transporters)	-5.6	0.032
	Consensus AI952772/5745082 /Hs.300865 immunoglobulin lambda	-8.3	0.017

Figure 1.
EGFR mRNA and protein expression *in vitro* in HuCCT1 and A431 cell lines. Cells were seeded in medium supplemented with 10% fetal bovine serum (FBS). When 50-60% confluency was reached they where serum starved for 24 hours, after which they were treated with growth media, erlotinib (5 μM), cetuximab (20 nM), or erlotinib (5 μM) plus cetuximab (20 nM). **A and B**) Erlotinib treatment induces a steady increase in EGFR mRNA, that is followed by augmented protein expression. **C and D)** In A431 cells there is no increase in EGFR mRNA after treatment with erlotinib. **E)** EGFR pathway activity after treatment with erlotinib, cetuximab, or the combination of both agents in HuCCT1 and A431 cell lines. After overnight serum-starvation cells were treated for 1, 6, and 24 hours. C, cetuximab; E, erlotinib; GM, growth media. mRNA and protein values are ratios normalized to their correlative control values, and represent mean ± SD of 4 determinations.



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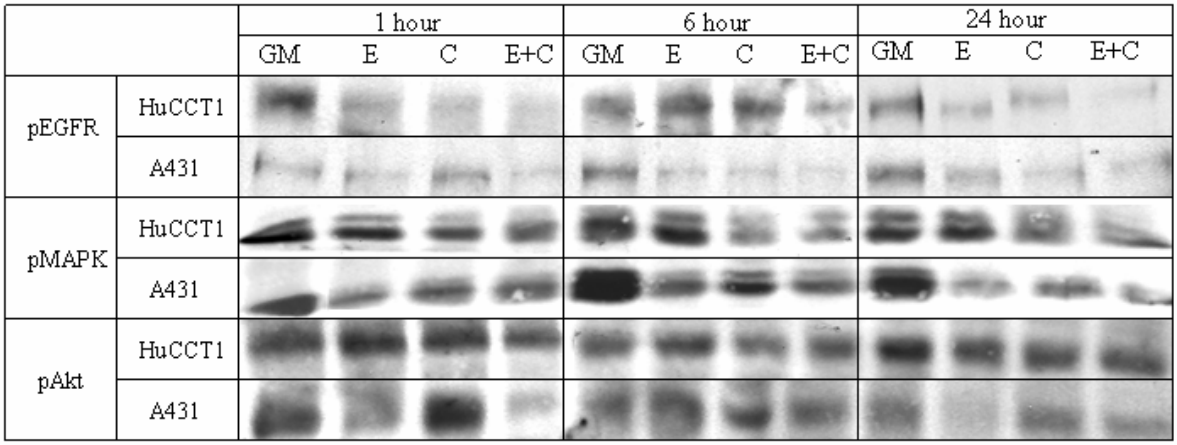


Figure 2.
Effect of 24 hours of treatment of HuCCT1 cells after being transfected with small interference RNA (siRNA) directed against the EGFR (gray bars, not transfected; black bars, transfected). Cells were plated in a 24-well plate at $5x10^4$ per well, and after 24 hours were transfected with siRNA and Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. Control consisted of HuCCT1 cells in the presence of the transfection reagent without siRNA, and a duplicate of the treatment arms without siRNA. After 24 hours the cells were treated with growth media, erlotinib (5 μM), cetuximab (20 nM), or erlotinib (5 μM) plus cetuximab (20 nM). **A)** EGFR mRNA synthesis, as assessed by real-time RT-PCR. The induction of EGFR synthesis induced by erlotinib is efficiently abrogated by siRNA. **B)** EGFR protein levels closely follow mRNA dynamics, as evidenced by the fact that siRNA induces a significant decrease in protein levels; cetuximab and siRNA have an additive effect in downregulating EGFR. **C)** The TUNEL assay in untransfected cells showed that, whereas erlotinib or cetuximab alone modestly increased the proportion of cells undergoing apoptosis (baseline 2.3%, erlotinib 9.7%, cetuximab 7.3%), the combination achieved a dramatic increase (54.1%) in cells undergoing programmed cell death. In siRNA-treated cells, however, maximal apoptosis was observed in erlotinib-treated cells. C, cetuximab; E, erlotinib; GM, growth media. mRNA values are normalized to baseline, and represent mean ± SD of 4 determinations. Protein values represent mean ± SD of 4 determinations. Apoptosis values represents mean ± SD of 2 determinations. * indicates $P < 0.05$ (t-test), comparing non-transfected versus transfected within treatment pairs; † indicates $P < 0.05$ (t-test), comparing GM versus different treatments within transfection groups.

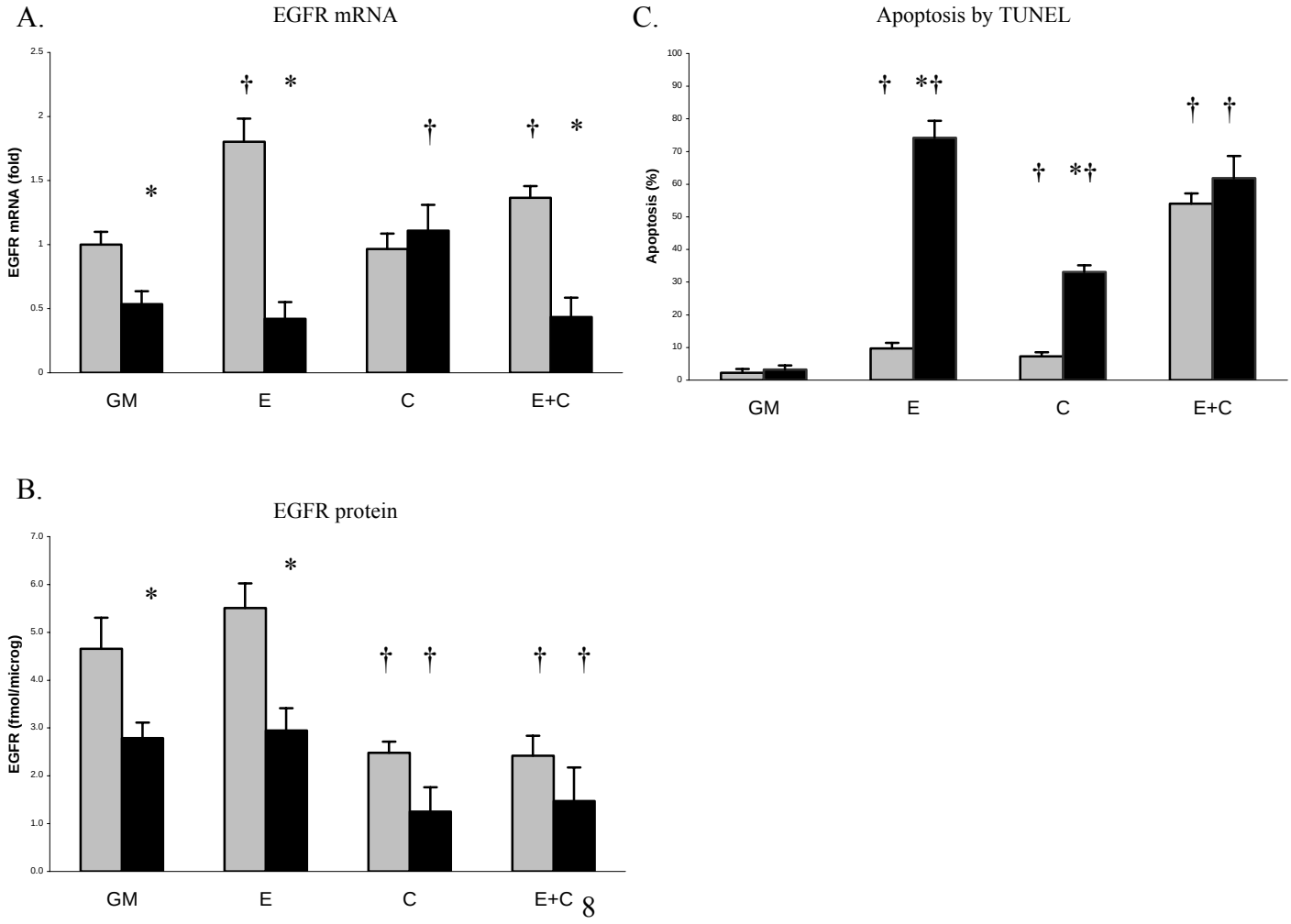
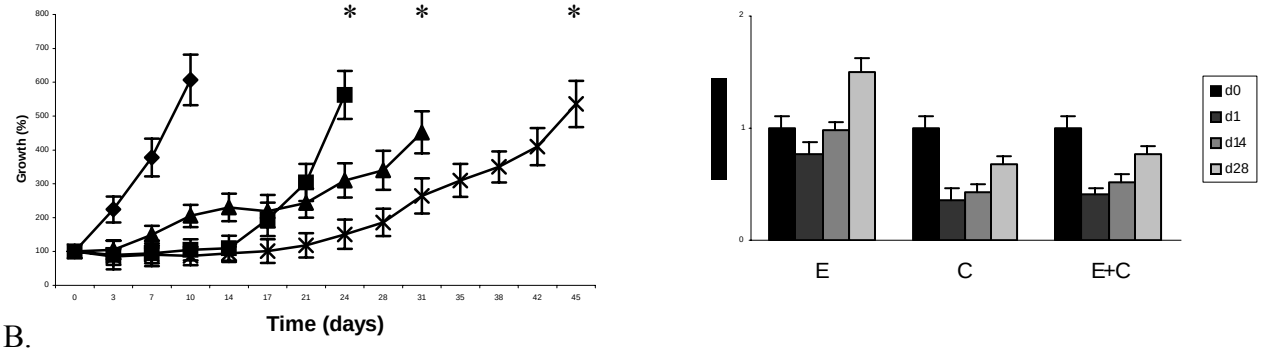


Figure 3.

A-C) Growth evaluation of A431 (A), HuCCT1 (B), and Panc430 (C) cell lines *in vivo*. Six-week-old female athymic nude mice (Harlan, IN, US) were used for this purpose. $5x10^6$ A431, HuCCT1, and Panc430 cells were injected subcutaneously in each flank. Tumors were grown to a size of 0.2 cm^3, and mice were stratified by tumor volume into different groups (6 mice [12 tumors] per group) that were treated with vehicle (♦), erlotinib (■), cetuximab (▲), or a combination of both (×) during 14 days. Survival surgery was performed at baseline, and 1, 14, and 28 after starting therapy. Tumor samples were snap-frozen, and EGFR levels at each time point from all treatment groups were determined by ELISA. Left graphs show tumor growth plots. Right graphs show relative EGFR levels as assessed by ELISA. In HuCCT1 four doses of cetuximab were given after completion of erlotinib (arrow), and growth was significantly arrested. In Panc430 growth was significantly lower in the combination group only after 28 days of initiating therapy. **D-E).** Analysis of Ki67 in HuCCT1 and A431 mice xenografts. Growth values are expressed as percentage relative to baseline ± SD (n = 12 tumors per group). Protein values are ratios normalized to their correlative control values, and represent mean ± SD of 4 determinations. C, cetuximab; E, erlotinib. * indicates $P < 0.05$ (t-test), compared with control.

A.



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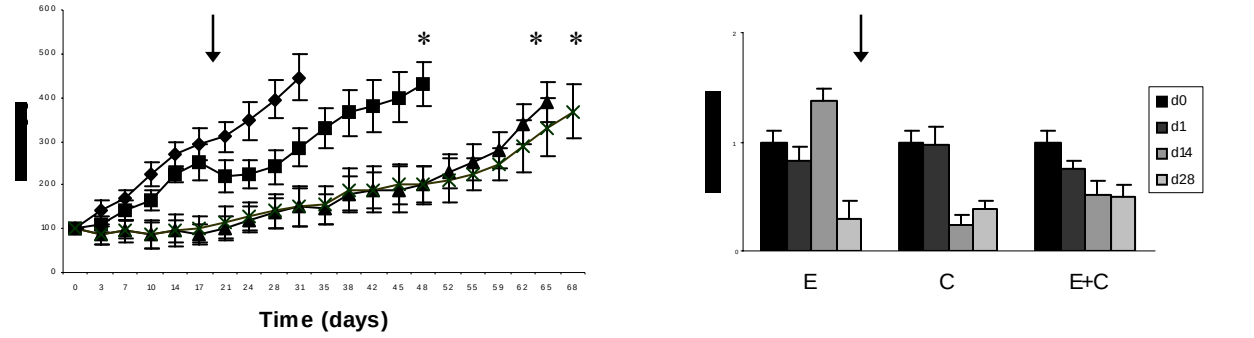

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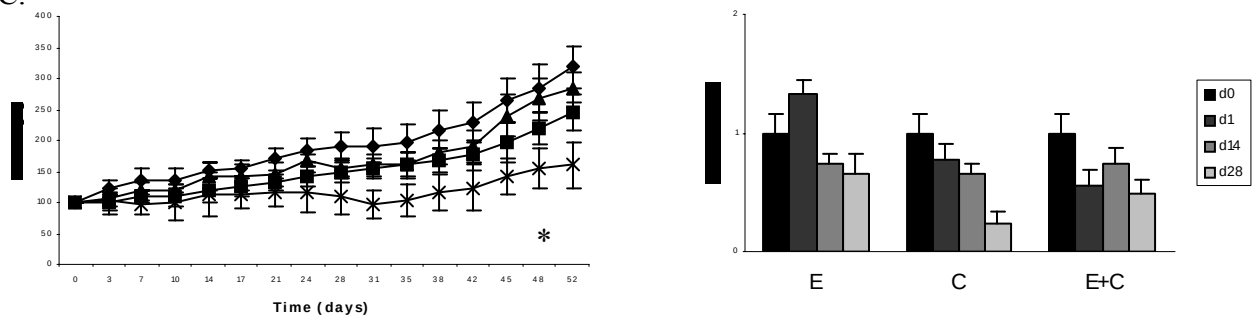
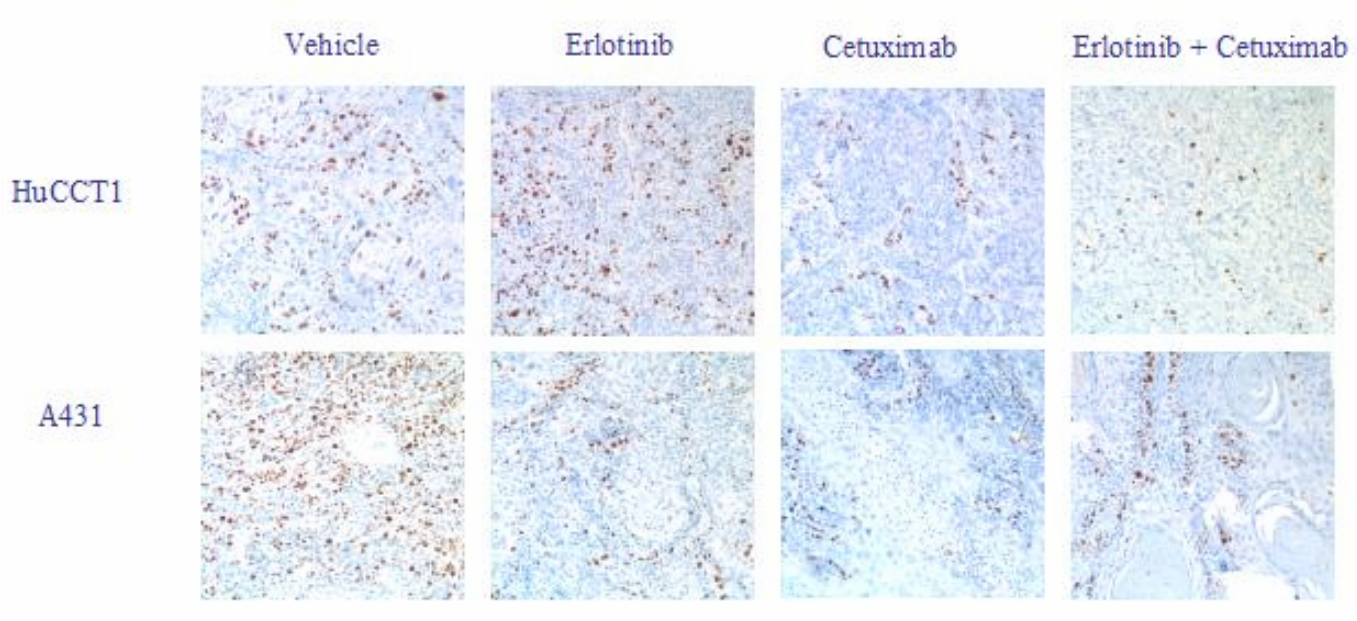


D. Ki67


Vehicle
Erlotinib
Cetuximab
Erlotinib + Cetuximab
HuCCT1
A431

APPENDIX B: ADDITIONAL MATERIAL AND METHODS INFORMATION

Drugs

Erlotinib was provided by OSI Pharmaceuticals (Melville, NY). Cetuximab was provided by ImClone Systems (New York, NY).

Western Blot analysis

Following treatment during 1, 6, and 24 hours cells were harvested. Equal amounts of protein (50 μgr) were resolved on 10% polyacrilamide gels. Gels were transferred onto nitrocellulose membranes that were incubated overnight at 4^{o}C with antibodies against phospho-EGFR, phospho-MAPK, and phospho-Akt (#2232, #2234, #9271, and #9101, respectively; Cell Signaling Technology, Beverley, MA). The immunoreactive proteins were detected using the enhanced chemiluminescence method (ECL) (Amersham, Piscataway, NJ).

Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from cell pellets using the RNeasy™ Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Relative quantification of EGFR mRNA was achieved using an iCycler iQ real-time PCR detection system (Bio-Rad) with Sybr green as the fluorophore (Bio-Rad). Primer sequences used for EGFR were used as previously published (20). Accumulation of the specific PCR products was detected as an increase in fluorescence that was plotted against cycle number to determine the CT values. Relative expression (RE) of the mRNA analyzed was estimated using the formula: $RE = 2^{-\Delta CT}$, where ΔCT = CT (mRNA) – CT (GAPDH).

ELISA Assay

An immunoezymatic assay (ELISA) was used for quantification of EGFR (Oncogene Research Products, San Diego, CA) following the manufacturer's instructions.

TUNEL assay

Quantification of apoptosis was assessed in duplicate by the terminal deosynucleotidyl transferase nick-end-labeling (TUNEL) technique, using a kit commercially available (Guava®TUNEL Kit, Guava Technologies, Hayward, CA).

Immunohistochemical analysis

Tumors were fixed and paraffin embedded using standard procedures. 5 micron sections were stained after citrate-steam antigen retrieval with Ki67 (M7187; DAKO, Carpinteria, CA) and EGFR (28-0005; Zymed, San Francisco, CA) primary antibodies. A biotinilated secondary antibody was used, followed by streptavidin conjugated HRP and DAB chromogen (K0690; DAKO).

APPENDIX C: REFERENCES

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