Cetuximab preclinical antitumor activity (monotherapy and combination based) is not predicted by relative total or activated epidermal growth factor receptor tumor expression levels


Bristol-Myers Squibb Co., Pharmaceutical Research Institute, Oncology Drug Discovery, Princeton, New Jersey

Abstract

Although Erbitux (cetuximab) has proven therapeutic benefit in the clinical setting, the molecular determinants predicting responsiveness to this agent are still not very well understood. Here, we assessed the relationship between basal total and activated (pY1068) epidermal growth factor receptor (EGFR) levels in a tumor and the responsiveness to cetuximab monotherapy or combination-based treatment using human xenograft models. Cetuximab treatment alone (0.25 – 1 mg/mouse/injection, q3d, i.p.) effectively delayed the growth of GEO and L2987 tumors by a minimum of 10 days corresponding to log cell kill values of ≥1.0. Borderline activity was seen in the A549 and WiDr xenografts. However, cetuximab failed to show any significant antitumor activity in the HT29, HCT116, LOVO, Colo205, LX-1, HCC70, and N87 models. All of the studied tumors had detectable yet variable levels of EGFR. For combination regimens, cetuximab (1 mg/mouse/injection, q3dx5, i.p.) and cisplatin (4.5 mg/kg/injection, q3dx5, i.v.) proved to be significantly more efficacious than individual monotherapies in the cisplatin-refractory yet cetuximab-responsive GEO tumor model (P < 0.001). However, no therapeutic enhancement was observed in the cisplatin and cetuximab weakly responsive A549 xenograft. Similarly, combinations of CPT-11 (48 mg/kg/injection, q3dx5, i.v.) with cetuximab (1 mg/mouse/injection, q3dx5, i.p.) failed to show any improvements over individual monotherapies in the cetuximab resistant/weakly responsive HT29, A549, and WiDr models. We conclude that preclinical activity associated with cetuximab monotherapy does not correlate directly with relative basal levels of total or activated (pY1068) EGFR in a tumor. Moreover, robust single-agent activity by cetuximab may be the best predictor for this agent to potentiate chemotherapy-mediated antitumor activities. [Mol Cancer Ther 2006;5(1):104 – 13]

Introduction

Erbitux (cetuximab) is a chimeric mouse/human monoclonal antibody of the IgG1 subclass that targets the human epidermal growth factor receptor (EGFR; ref. 1). EGFR is overexpressed in about one third of all human cancers and has been directly implicated in tumor growth and progression (1). In particular, elevated expression levels of EGFR in colon carcinomas have been linked to more aggressive disease and poor prognosis (2). Hence, strategies aimed at disrupting the function of the EGFR have been investigated as exciting new approaches for the treatment of cancer and include small molecule inhibitors as well as various biologics approaches (3). In the case of the monoclonal antibody cetuximab, clinical studies have shown that this agent is capable of significantly inhibiting tumor growth (4). In particular, about 11% to 23% of irinotecan (CPT-11) refractory, EGFR-positive colon cancer patients dramatically benefited from this antibody therapy when administered as a single agent or in combination with irinotecan (5). Thus, in February 2004, cetuximab was approved in the United States for the treatment of advanced colon cancer in combination with irinotecan. Nevertheless, several questions remain unanswered about this therapeutic. Specifically, it is still not very well understood why only a fraction of patients with EGFR-positive tumors respond to cetuximab treatment (i.e., which exact patient population will most likely benefit from this antibody therapy).

Preclinically, many studies have shown that treatment with cetuximab results in inhibition of tumor growth in nude mice bearing xenografts of human cancer cell lines (6–14). Moreover, treatment with the antibody in combination with chemotherapeutic drugs or radiation has been reported to be more efficacious than individual monotherapies in the same or similar xenograft model systems (6–11, 13–20). Although published preclinical reports suggest that EGFR expression may be needed for cetuximab activity (21, 22), recent clinical evidence indicates that response to cetuximab treatment is independent of the relative degree of EGFR expression levels in tumors (23).
The objective of the following studies was to further expand on our knowledge of cetuximab monotherapy activities in a broad panel of EGFR-positive human carcinoma xenografts. In particular, we wanted to assess whether any correlation existed between basal total EGFR or basal activated (pY1068) EGFR levels in a tumor and the responsiveness to cetuximab treatment. To this effect, we employed tumor models from several different histologies (colon, lung, breast, and gastric cancer), all known to frequently overexpress EGFR (24). Finally, we also evaluated the utility of combining cetuximab with the chemotherapeutic agents irinotecan (CPT-11) or cisplatin in chemotherapy-refractory or poorly responding tumor models because positive clinical results have been reported in the same setting (5, 25). Based on our collective preclinical data, attempts were then made to identify the determinants that predict synergistic antitumor activity of cetuximab with conventional anticancer agents.

Materials and Methods

Compounds and Their Administrations

Clinical-grade cetuximab was supplied by ImClone Systems (New York, NY) at a concentration of 2 mg/mL in a buffer consisting of 10 mmol/L sodium phosphate and 145 mmol/L sodium chloride at pH 7.2. For all efficacy studies requiring lower concentrations of cetuximab, the stock solution was diluted with sterile PBS (pH 7.4). Cetuximab was administered i.p. at a constant volume of 0.5 mL/mouse. Clinical-grade cisplatin (Platinol) was obtained from Bristol-Myers Squibb (Princeton, NJ), and irinotecan (CPT-11) was purchased commercially; they were both dissolved in sterile 0.9% NaCl (saline) and administered i.v. in a volume of 0.01 mL/g of mouse body weight. CPT-11 and cisplatin were administered up to their maximum tolerated dose (MTD) as previously reported (26, 27).

Chemicals and Reagents

Complete protease inhibitor tablets were from Roche Diagnostics (Indianapolis, IN). MicroBCA reagents were from Pierce (Rockford, IL). Human-specific EGFR and pY1068 EGFR ELISA kits were purchased from Biosource International, Inc. (Camarillo, CA). All other chemicals and reagents were from Sigma (St. Louis, MO). Unless otherwise specified, sterile buffers and solutions were obtained from Life Technologies (Carlsbad, CA). Sterile tissue culture ware was obtained from Fisher Scientific Co. (Hanover Park, IL).

Animals

Female athymic, nude mice (5–6 weeks of age) were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) and maintained in an ammonia-free environment in a defined and pathogen-free colony. Animals were quarantined for ~1 week before their use for tumor propagation and drug efficacy testing. They were fed food and water ad libitum. All studies were done in accordance with Bristol-Myers Squibb and the American Association for Accreditation of Laboratory Animal Care guidelines.

Tumor Lines

All human carcinoma xenografts were propagated in nude mice as s.c. transplants using tumor fragments obtained from donor mice. The models employed included the colon carcinomas: GEO, HT29, HCT116, LOVO, WiDr, and Colo205; the lung carcinomas: L2987, A549, and LX-1; the breast carcinoma, HCC70; and the gastric carcinoma, N87. Tumor passage occurred approximately every 2 to 4 weeks depending on the model. All tumor implants for efficacy testing were s.c. in nude mice and employed tumor fragments from tumors passaged at least twice in vivo.

Quantitation of Total EGFR and Activated (pY1068) EGFR Levels in Human Tumor Xenograft Samples

Human tumor xenografts were established in athymic mice and surgically removed when they reached an average tumor size of 150 to 300 mg. Resected tumors were immediately snap-frozen in liquid nitrogen and stored at ~80°C until further use. Frozen tumor tissues were then lysed in ice-cold lysis buffer that contained 10% Triton X-100, 5% glycerol, 20 mmol/L Tris-HCl (pH 7.7), 0.5 mmol/L EDTA, 0.1 mol/L NaCl, 1 mmol/L sodium orthovanadate, 1% Phosphatase Inhibitor Cocktail 2 (Sigma), and 2% Complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). The sample was subsequently homogenized using a Wharton tissue homogenizer. Cell lysates were clarified by centrifugation (10 minutes at 10,000 × g), and protein concentrations were determined using the MicroBCA method (Pierce). Tumor lysates from all of the samples were adjusted to a concentration of 3 mg/mL protein. Equal amounts of total protein (30 µg) from each tumor sample were then used to analyze the relative total human EGFR and activated EGFR (pY1068) levels in the different xenograft samples as determined by ELISA assay according to the protocol recommended by the manufacturer (Biosource International). Expected values for different tumor cell lines, as provided by the assay manufacturer, were used as a guideline to classify samples as high, medium, or low EGFR expressers. Furthermore, we considered samples with EGFR levels higher than the calculated mean for all evaluated tumor samples (~20 ng/mL) as high receptor expressers.

In vivo Antitumor Testing

For each experiment, athymic mice were implanted s.c. with ~1 mm³ tumor fragments obtained from donor animals. Tumors were allowed to grow to a size between 100 to 200 mg, and animals were then distributed to various treatment and control groups (n = 8 per group). Treatment of each animal was based on individual body weight on a mg per kg (mg/kg) basis for all drugs except cetuximab, which was administered on a mg per mouse (mg/mouse) basis delivered in 0.5 mL/mouse. Treated animals were checked daily for treatment related toxicity/mortality. In addition, average body weights for each group were determined before the initiation of treatment (Wt1) and following the last treatment dose (Wt2). The difference of body weight (Wt2 – Wt1) was then used to estimate the degree of treatment-related toxicity. Tumors were...
measured with a caliper twice a week, until they reached a predetermined “target” size (0.5–1 g depending on tumor model used). Tumor weights (mg) were estimated from the following formula:

\[ \text{Tumor weight (mg)} = \left\{ \text{length (mm)} \times \text{width (mm)} \right\}/2 \]

Efficacy of antitumor agents was determined in two ways:

(a) Calculating the relative % tumor growth inhibition (%TGI) of treated animals at different time points using the formula:

\[ \% \text{TGI} = \left( \frac{C_t - T_t}{C_0 - C_0} \right) \times 100 \]

where, tumor volume doubling time (TVDT) = median time (days) required for the treated tumors (T) to reach a predetermined target size compared with those of the control group (C). Antitumor efficacy could then be compared between different treatment groups by estimating the overall gross log cell kill (LCK). The following formula was employed to calculate the LCK:

\[ \text{LCK} = T - C / (3.32 \times \text{TVDT}) \]

where, tumor volume doubling time (TVDT) = median time (days) for control tumors to reach target size – median time (days) for control tumors to reach half the target size.

Antitumor activity was defined as a statistically significant LCK of ≥1.0 (19). Borderline activity, particularly for monotherapy experiments employing the cytostatic agent cetuximab, was defined as a continuous tumor growth inhibition (%TGI) of treated animals at different time points using the formula:

\[ \% \text{TGI} = \left( \frac{C_t - T_t}{C_0 - C_0} \right) \times 100 \]

(b) Calculating the tumor growth delay (T-C value), defined as the difference in time (days) required for the treated tumors (T) to reach a predetermined target size compared with those of the control group (C). Antitumor efficacy could then be compared between different treatment groups by estimating the overall gross log cell kill (LCK). The following formula was employed to calculate the LCK:

\[ \text{LCK} = T - C / (3.32 \times \text{TVDT}) \]

where, tumor volume doubling time (TVDT) = median time (days) for control tumors to reach target size – median time (days) for control tumors to reach half the target size.

Antitumor activity was defined as a statistically significant LCK of ≥1.0 (19). Borderline activity, particularly for monotherapy experiments employing the cytostatic agent cetuximab, was defined as a continuous tumor growth inhibition (%TGI) of treated animals at different time points using the formula:

\[ \% \text{TGI} = \left( \frac{C_t - T_t}{C_0 - C_0} \right) \times 100 \]

Results

EGFR Expression Levels of Tumor Xenografts

Cetuximab was evaluated for its antitumor activity in a panel of 11 different human tumor xenografts with various EGFR expression levels. The employed tumor models were maintained in vivo for a minimum of two passages before use in an experiment and included six colon carcinomas (LOVO, WiDr, GEO, HCT-116, Colo-205, and HT-29), three lung carcinomas (L2987, A549, and LX-1), one breast carcinoma (HCC70), and one gastric carcinoma (N87). EGFR expression levels were determined in tumor lysates via ELISA (specific for human EGFR) and ranged from high (>20 ng/mL) in L2987, HCC70, and LOVO; to medium (10–20 ng/mL) in WiDr, GEO, A549, LX-1, and HCT116; to low (<10 ng/mL) in Colo205, N87, and HT29 (Fig. 1A). All samples were above the limit of EGFR detection.

Cetuximab Monotherapy Antitumor Activity

Cetuximab administrated i.p. at 1 mg/mouse/injection on an intermittent dosing schedule has been successfully used in a number of xenograft models (6–8, 11, 13, 19, 20, 30, 31). However, a systematic evaluation of the optimal dose and schedule of this antibody in preclinical models has not been described. Moreover, a comprehensive and simultaneous analysis of the antitumor activity of this antibody in a large panel of tumor models has not been reported.

Schedule comparison of 1 mg/mouse/injection in the EGFR moderately expressing A549 tumor model showed that there was no appreciable difference in efficacy when the antibody was administered either on a daily (qdx13), every other day (q2dx7), or every 3-day (q3dx5) schedule (data not shown). Moreover, a dose response experiment using a q3dx5 schedule in the GEO colon carcinoma model showed that cetuximab was equally effective at dose levels between 0.25 and 1 mg/mouse/injection, i.p., every 3 days (q3d) for a minimum of five injections represented a likely optimal treatment regimen, which was then applied to profile the antitumor activity of the antibody in our large panel of EGFR-positive xenograft models.

Using this preclinically defined optimal treatment regimen, cetuximab showed marked and reproducible antitumor activity in the L2987 (human lung carcinoma) and GEO (human colon carcinoma) models, achieving continuous tumor growth inhibitions in excess of 50% for at least 1 TVDT. This was accompanied by a significant tumor growth delay corresponding to >1 LCK in the majority of experiments. Responses synonymous with borderline activity based on tumor growth inhibition (%TGI ≥ 50% for 1 TVDT, accompanied with a statistically significant tumor growth delay) were occasionally seen in the A549 (human lung carcinoma) and WiDr (human colon carcinoma) xenograft models. However, the antibody was completely inactive in the HT29, HCT116, LOVO, LX-1, HCC70, N87, and Colo205 human carcinoma models, where neither significant tumor growth inhibitions nor considerable tumor growth delays were observed. In addition, longer treatment durations did not seem to affect the antitumor responses in the cetuximab-resistant and cetuximab-borderline sensitive...
xenografts, whereas extended dosing periods prolonged tumor growth inhibitions in the GEO and L2987 cetuximab-sensitive tumor models. These results are summarized in Table 1.

Lack of Correlation between Basal Levels of Total EGFR or Relative Activated EGFR (pY1068) and Antitumor Response to Cetuximab Treatment

We also assessed the relationship between the basal levels of EGFR expression in the 11 human carcinoma xenografts and their responses to cetuximab monotherapy, as measured by the maximum LCK achieved on a q3dx5 schedule (or a q3dx10 schedule for the N87 and HCC70 tumor models because other schedules were not evaluated). In the cetuximab-responsive GEO and L2987 tumors, maximum LCK values of 1.4 and 1.3, respectively, were achieved. In addition, tumor growth inhibitions in excess of 50% for at least 1 TVDT were attained in 100% of the experiments involving these two tumor models (Table 1). In the borderline cetuximab-sensitive A549 and WiDr xenografts, maximum LCK values of only 0.7 and 0.3, respectively, were obtained. Moreover, mean TGIs in excess of 50% for at least one TVDT were achieved in only two of four experiments for the WiDr xenograft and four of six experiments for the A549 tumor model (Table 1). Finally, in the cetuximab-resistant models, maximum LCK values rarely went higher than 0.1, and %TGI values were always below levels sufficient to be defined as borderline active (Table 1). ELISA analysis of human EGFR expression showed a >10-fold difference in the levels of EGFR protein among these tumors (Fig. 1A). Upon examination, the levels of EGFR expression did not correlate significantly with tumor response to cetuximab treatment (r = 0.38238, P = 0.2458; Fig. 1B). Although L2987 had the highest levels of EGFR expression among the tested xenograft samples (61.23 ng/mL) and showed one of the most sensitive phenotypes to cetuximab treatment (maximum 1.3 LCK), other high expressers of EGFR, such as HCC70 and LOVO (40.55 and 27.77 ng/mL, respectively), failed to respond effectively to cetuximab therapy (maximum LCK values achieved were 0.1 and 0.2 LCK, respectively).

Conversely, GEO reproducibly showed significant sensitivity to cetuximab treatment (Table 1), yet this tumor model expressed a relatively low level of total basal EGFR (10.88 ng/mL; Fig. 1A).

Figure 1. A, basal total EGFR expression levels in various xenograft tumor samples. An equal amount of total protein (30 μg) from each tumor sample was used to analyze the relative total EGFR levels in the different xenograft samples by ELISA. B, basal total EGFR levels versus antitumor efficacy of cetuximab (maximum LCK achieved at an optimal dose using the q3dx5 schedule, or q3dx10 for the N87 and HCC70 models since other schedules were not tested). Basal total EGFR levels were plotted versus the maximum antitumor efficacy obtained in each individual xenograft model. A linear regression analysis was done to determine degree of correlation between these two data sets (solid line). Dotted lines, 95% confidence interval of the linear regression line. C, basal relative activated EGFR (pY1068/Total EGFR) versus cetuximab responsiveness. Basal activated EGFR (pY1068) levels were normalized to basal total EGFR levels to determine basal "relative" activated EGFR levels. A one-way ANOVA showed that there was no significant difference in basal relative activated EGFR levels between cetuximab-sensitive (GEO, L2987, A549, and WiDr) and cetuximab-resistant (HCT116, HT29, LOVO, Colo205, N87, HCC70, and LX-1) tumor models (P = 0.19662).
We also quantitated the basal levels of activated EGFR (pY1068) in the tumor xenografts by ELISA. Considerable levels were detected in all but two models (i.e., A549 and HT29 tumor xenografts). Upon analysis, there was no significant difference in relative activated EGFR levels (pY1068/total EGFR) among the four cetuximab-sensitive/borderline-sensitive tumor models (L2987, GEO, A549, and WiDr) and the seven cetuximab-resistant xenografts (Colo205, LX-1, HT29, HCT116, N87, HCC70, and LOVO) as determined by one-way ANOVA ($F = 1.9447$, $P = 0.19662$; Fig. 1C). Responsiveness to cetuximab was also independent of absolute values of activated (pY1068) EGFR as well as TVDT (data not shown).

Cetuximab In vivo Antitumor Activity (Combination Therapy)

Antitumor activity of cetuximab in combination with the cytotoxic agents cisplatin or CPT-11 was assessed in a series of chemotherapy refractory (i.e., resistant to treatment) or poorly responding human tumor models. Specifically, we evaluated cetuximab in combination with cisplatin in the GEO and A549 cisplatin-refractory tumor models and cetuximab combined with CPT-11 in the HT29, A549, and WiDr CPT-11 poorly responding/refractory xenografts. In these combination studies, all test agents were administered using an every 3 days schedule (q3dx5), and a tumor end point of 1 g was used unless otherwise specified. In addition, cetuximab treatment always followed, within 15 minutes, the cytotoxic drug administration. All combination treatments were well tolerated without significant toxicity.

As previously described, cetuximab monotherapy reproducibly showed antitumor activity in the GEO human tumor xenograft (Table 1). In contrast, cisplatin monotherapy was ineffective in this colon carcinoma model at all tested dose levels, including its near MTD of 4.5 mg/kg injection (Table 2). Interestingly, combination regimens

<table>
<thead>
<tr>
<th>Tumor (type)</th>
<th>EGFR status</th>
<th>Experiment no.</th>
<th>Dose (mg/mouse/injection)</th>
<th>Route, schedule</th>
<th>LCK at tumor size 0.5 g</th>
<th>1.0 g</th>
<th>≥50 %TGI for 1 TVDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEO (colon)</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx10,14</td>
<td>1.8^†</td>
<td>2.7^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>i.p., q3dx5,14</td>
<td>1.2^†</td>
<td>0.8^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.25</td>
<td>i.p., q3dx5,11</td>
<td>1.1^†</td>
<td>1.4^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.25</td>
<td>i.p., q3dx6,10</td>
<td>1.3^†</td>
<td>1.3^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.25</td>
<td>i.p., q3dx4,10</td>
<td>1.0^†</td>
<td>0.8^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1</td>
<td>i.p., q3dx6,11</td>
<td>1.1^†</td>
<td>0.7^†</td>
<td>Yes</td>
</tr>
<tr>
<td>WiDr (colon)</td>
<td>++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx10,14</td>
<td>0.2</td>
<td>0.6</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>i.p., q3dx10,15</td>
<td>0.0</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.25</td>
<td>i.p., q3dx5,14</td>
<td>0.2</td>
<td>0.3^‡</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td>i.p., q3dx6,19</td>
<td>0.2^‡</td>
<td>0.3^‡</td>
<td>Yes</td>
</tr>
<tr>
<td>HCT116 (colon)</td>
<td>++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx3,13</td>
<td>0.1</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>i.p., q3dx10,14</td>
<td>0.0</td>
<td>0.0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td>i.p., q3dx5,10</td>
<td>0.0</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>LOVO (colon)</td>
<td>++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx5,14</td>
<td>0.0</td>
<td>0.2</td>
<td>No</td>
</tr>
<tr>
<td>Colo205 (colon)</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx5,14</td>
<td>0.1</td>
<td>0.0</td>
<td>No</td>
</tr>
<tr>
<td>N87 (gastric)</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx10,16</td>
<td>0.1</td>
<td>0.0</td>
<td>No</td>
</tr>
<tr>
<td>HCC70 (breast)</td>
<td>+++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx10,28</td>
<td>0.1</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td>L2987 (lung)</td>
<td>+++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx5,16</td>
<td>1.0^†</td>
<td>1.3^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.5</td>
<td>i.p., q3dx4,22</td>
<td>0.9</td>
<td>0.8^†</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td>i.p., q3dx6,20</td>
<td>1.0^‡</td>
<td>1.3^‡</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td>i.p., q3dx6,23</td>
<td>2.4^†</td>
<td>2.4^†</td>
<td>Yes</td>
</tr>
<tr>
<td>LX-1 (lung)</td>
<td>++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx5,14</td>
<td>0.3</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>++</td>
<td>1</td>
<td>1</td>
<td>i.p., q3dx10,18</td>
<td>0.4^‡</td>
<td>0.6</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>i.p., q3dx5,26</td>
<td>0.5</td>
<td>0.7^‡</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td>i.p., q3dx3,21</td>
<td>0.1</td>
<td>0.3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td>i.p., q3dx5,22</td>
<td>0.1</td>
<td>0.3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1</td>
<td>i.p., q3dx5,21</td>
<td>0.7</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1</td>
<td>i.p., q3dx6,21</td>
<td>1.0^‡</td>
<td>ND</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NOTE: LCK numbers in bold illustrate maximum antitumor activity obtained in each tumor model using a q3dx5 schedule (q3dx10 for N87 and HCC70). LCK values were then plotted versus total EGFR levels to correlate these two variables (see Fig. 1B).

Abbreviation: ND, not determined.

*EGFR receptor levels: +++ (>20 ng/mL), ++ (10-20 ng/mL), + (<10 ng/mL).
^Statistically significant at $P < 0.001$ versus untreated control.
Statistically significant at $P < 0.05$ versus untreated control.
Statistically significant at $P < 0.01$ versus untreated control.
of cetuximab and cisplatin clearly showed improved antitumor activities over their respective monotherapies. For instance, the combination of 1 mg/mouse/injection of cetuximab with 4.5 mg/kg/injection cisplatin resulted in a LCK of 1.6 versus a maximum LCK of 0.8 for cetuximab monotherapy ($P < 0.001$ versus untreated control and cetuximab monotherapy; Fig. 2; Table 2). Moreover, higher doses of cisplatin were associated with better antitumor response rates in the cetuximab plus cisplatin combination regimens, although cisplatin treatment by itself did not show any activity (Table 2).

We also attempted to extend the observation of therapeutic synergy obtained in the GEO model to another cisplatin-refractory xenograft tumor, the A549 human lung carcinoma. As described previously, cetuximab monotherapy was at best borderline active in some of the experiments employing this tumor model (Table 1). In this particular combination study with cisplatin, cetuximab monotherapy at 1 mg/mouse/injection (q3dx5, i.p.) failed to achieve mean tumor growth inhibitions in excess of 50% for at least one continuous TVDT resulting in a 0.3 LCK. Cisplatin at an MTD of 6 mg/kg/injection was likewise ineffective (0.3 LCK). Moreover, when cetuximab therapy was added to the cisplatin regimen, there was no significant increase in antitumor activity over the monotherapy regimens. Hence, combinations of these two agents did not improve the overall efficacy profile in the A549 human lung carcinoma model. These results are summarized in Table 2.

Combinations of cetuximab with CPT-11 were tested initially in the HT29 human colon carcinoma model. As described previously (Table 1), cetuximab monotherapy was inactive in this tumor model. CPT-11 monotherapy

### Table 2. Preclinical antitumor activity of cetuximab plus cisplatin combination therapy

<table>
<thead>
<tr>
<th>Tumor (type)</th>
<th>Cetuximab dose (mg/mouse/injection), i.p.</th>
<th>Cisplatin dose (mg/kg/injection), i.v.</th>
<th>Schedule</th>
<th>LCK at tumor target size of 1 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEO (colon)</td>
<td>1</td>
<td>—</td>
<td>q3dx5,14</td>
<td>0.8*</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>4.5</td>
<td>q3dx5,14</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.5</td>
<td>q3dx5,14</td>
<td>1.6*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>q3dx5,14</td>
<td>1.6*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>q3dx5,14</td>
<td>1.4*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4.5</td>
<td>q3dx5,14</td>
<td>2.0*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3</td>
<td>q3dx5,14</td>
<td>1.5*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
<td>q3dx5,14</td>
<td>1.5*</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>4.5</td>
<td>q3dx5,14</td>
<td>1.6*</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3</td>
<td>q3dx5,14</td>
<td>1.5*</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>2</td>
<td>q3dx5,14</td>
<td>1.1*</td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>1</td>
<td>—</td>
<td>q3dx5,21</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>6</td>
<td>q3dx5,21</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6</td>
<td>q3dx5,21</td>
<td>0.5*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.5</td>
<td>q3dx5,21</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>q3dx5,21</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>6</td>
<td>q3dx5,21</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>4.5</td>
<td>q3dx5,21</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3</td>
<td>q3dx5,21</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Statistically significant at $P < 0.001$ versus untreated control.

*Statistically significant at $P < 0.01$ versus the most effective monotherapy regimen tested (cetuximab for GEO; cisplatin for A549).

*Statistically significant at $P < 0.05$ versus the most effective monotherapy regimen tested (cetuximab for GEO; cisplatin for A549).

*Statistically significant at $P < 0.01$ versus untreated control.

*Statistically significant at $P < 0.05$ versus untreated control.

![Figure 2. Preclinical antitumor activity of cetuximab plus cisplatin combination chemotherapy versus s.c. GEO human colon carcinoma. Horizontal line, 1 LCK at tumor target size of 1.0 g. Arrows, days of drug treatment.](mct.aacrjournals.org)
inhibited HT29 tumor growth to a borderline active rating. For example, a LCK of 0.7 ($P < 0.01$ versus untreated control) was achieved for a tumor target size of 1.0 g at the MTD of 48 mg/kg/injection. However, adding cetuximab therapy to CPT-11 failed to significantly increase antitumor activity over CPT-11 monotherapy, and no synergy was attained in this tumor model (Table 3).

We also tested CPT-11 and cetuximab combinations in the A549 human lung carcinoma model. As described previously, mean tumor growth inhibitions in excess of 50% for at least one continuous TVDT period were achieved for cetuximab treatment in only four of six experiments, one of which was this particular combination therapy experiment. Cetuximab single-agent administration was borderline active and achieved a tumor growth delay corresponding to 0.7 LCK ($P < 0.05$ versus untreated control). CPT-11 monotherapy was inactive at its MTD of 48 mg/kg/injection (0.3 LCK). However, adding CPT-11 to cetuximab therapy failed to significantly increase antitumor activity over the cetuximab monotherapy regimen (Table 3).

Lastly, cetuximab and CPT-11 combination regimens were evaluated in the WiDr human colon carcinoma tumor model. As described previously (Table 1), cetuximab has weak and only occasional borderline activity in this xenograft model. In this particular combination therapy experiment, cetuximab was inactive and failed to achieve mean tumor growth inhibitions in excess of 50% for at least one continuous TVDT period. In contrast, CPT-11 was active, achieving 1.1 LCK at its MTD of 48 mg/kg/injection ($P < 0.001$ versus untreated control). Adding cetuximab to CPT-11 failed to significantly increase antitumor activity over CPT-11 monotherapy (Table 3).

### Discussion

Cetuximab monotherapy showed unequivocal activity in 2 of 11 evaluated human xenograft tumor models (L2987, human lung carcinoma; Geo, human colon carcinoma). Modest levels of tumor growth suppression were seen on occasion in two additional models, WiDr (human colon carcinoma) and A549 (human lung carcinoma). In contrast, cetuximab failed completely to show any antitumor activity in the majority of evaluated EGFR-positive tumors, including Colo205 (human colon carcinoma), HCC70 (human breast carcinoma), HCT116 (human colon carcinoma), LOVO (human colon carcinoma), N87 (human gastric carcinoma), LX-1 (human lung carcinoma), and HT29 (human colon carcinoma). Although tumor EGFR positivity may be a prerequisite for achieving antitumor activity with cetuximab, our data suggests that the relative tumor receptor levels do not predict responsiveness to the antibody and do not correlate with cetuximab efficacy. Moreover, basal levels of relative activated EGFR (pY1068 EGFR/total EGFR) do not directly correlate with the status of sensitivity to cetuximab therapy. These results draw multiple parallels to the clinical experience with cetuximab.

### Table 3. Preclinical antitumor activity of cetuximab plus CPT-11 combination therapy

<table>
<thead>
<tr>
<th>Tumor (type)</th>
<th>Cetuximab dose (mg/mouse/injection), i.p.</th>
<th>CPT-11 dose (mg/kg/injection), i.v.</th>
<th>Schedule</th>
<th>LCK at tumor target size of 1 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29 (colon)</td>
<td>1</td>
<td>48</td>
<td>q3dx5,13</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>1</td>
<td>48</td>
<td>q3dx5,26</td>
<td>0.7*</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WiDr (colon)</td>
<td>1</td>
<td>48</td>
<td>q3dx5,14</td>
<td>1.1*</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant at $P < 0.01$ versus untreated control.

†Statistically significant at $P < 0.05$ versus untreated control.

‡Statistically significant at $P < 0.001$ versus untreated control.
where monotherapy activity with this antibody has been likewise low, showing ~11% overall response rates in EGFR-positive, irinotecan-refractory metastatic colorectal cancer patients (5). Although increased gene copy number for EGFR may correlate with clinical response to cetuximab (32), others have reported that lower mRNA levels of EGFR may provide a better predictive marker of positive clinical outcome in colorectal cancer (33). Furthermore, clinical observations showed that response to cetuximab was not related to the relative strength of EGFR protein expression of the tumor specimen (23). In fact, one report even showed that cetuximab had activity in colorectal cancer patients with tumors that do not express the EGFR protein at all as determined by current immunohistochemistry techniques (34). Therefore, it is likely that molecular events other than basal EGFR levels (total and/or activated) are the true determinants of the tumor responsiveness to cetuximab treatment. For example, the dynamics of the EGFR activation status in tumors (i.e., changes in EGFR activation and its associated downstream pathway components upon antibody treatment) may have a much better correlation with efficacy and sensitivity to cetuximab treatment, which warrants further investigation.

Interestingly, clinical data suggest that response to the small molecule EGFR tyrosine kinase inhibitors, gefitinib (Iressa) and erlotinib (Tarceva), may be dependent on the number of EGFR gene copies and protein expression levels (35, 36). Moreover, recent reports show that somatic mutations in the tyrosine kinase domain of the EGFR gene significantly correlated with the clinical response to gefitinib in patients with chemotherapy-refractory non–small cell lung cancer (37, 38). Specifically, nearly all gefitinib-responsive patients harbored mutations in the EGFR kinase domain, whereas no mutations have been identified in nonresponsive, refractory cases. The main EGFR mutations identified in these studies included missense mutations (L858R) and in-frame deletions (L747-P753) in the kinase domain, which corresponded with enhanced EGFR and downstream pathway activation and were associated with increased sensitivity to inhibition by gefitinib in vitro compared with wild-type receptor (37–39). A similar scenario has been reported for the related small molecule ATP-competitive EGFR tyrosine kinase inhibitor, erlotinib (Tarceva), where five of seven tumors sensitive to erlotinib had somatic mutations in EGFR analogous to gefitinib; however, all of 10 erlotinib-refractory tumors were mutation free (40, 41). Although, Pao et al. showed some differences in EGFR activation among the mutants compared with other published data, the relative changes in sensitivity to the two small molecule inhibitors were similar (i.e., changes in sensitivity to erlotinib tracked with changes in sensitivity to gefitinib).

Preliminary evidence suggests that the discussed EGFR mutations associated with sensitivity to the small molecule inhibitors are not required for response to cetuximab nor do they seem predictive of response to cetuximab in the clinical setting (42, 43). This may be due in part to the fact that the sequence alterations published for gefitinib and erlotinib sensitivity cluster around the active site of the kinase, presumably stabilizing the interaction between the EGFR-tyrosine kinase domain and its competitive inhibitor (e.g., gefitinib and erlotinib; ref. 44). Because cetuximab is interacting with the extracellular domain of the receptor, it is likely that the antibody will act independently of these mutations. Thus, antibody-based EGFR inhibitors may have different overall activity profiles compared with gefitinib and erlotinib, although they all target the same molecular entity/pathway. Consequently, cetuximab, and perhaps other related antibodies targeting the extracellular domain of the EGFR, may have different molecular and genetic determinants that predict sensitivity compared with the small molecule inhibitors. Clearly, further studies are needed to fully address this question.

Previous reports have shown that cetuximab can potentiate antitumor effects of many cytotoxic agents, including cisplatin and CPT-11 (16, 45). However, few reports have tried to rationalize the determinants that predict therapeutic synergy. Here, we report that cetuximab and cisplatin combination regimens proved to be significantly more efficacious than either monotherapy alone in the cisplatin nonresponsive GEO human colon carcinoma tumor model. GEO tumors were also one of the most sensitive models tested for cetuximab monotherapy regimens. In contrast, the same cetuximab and cisplatin combination was without benefit in the cisplatin poorly responsive A549 human lung carcinoma model. In this particular experiment, cetuximab monotherapy was likewise inactive. Similarly, combinations of CPT-11 with cetuximab failed to show any clear improvements over the best monotherapy used in the CPT-11 weakly responsive HT29, A549, and WiDr tumor models. Both HT29 and WiDr experiments showed clear resistance to cetuximab monotherapy. Interestingly, in the A549 combination study, cetuximab alone approached single-agent activity, and the best antitumor effect occurred using a combination of cetuximab with CPT-11. Although this effect was not statistically different from the best monotherapy response, it may indicate a general trend. Collectively, our data suggest that robust cetuximab single-agent efficacy in a particular tumor may be a prerequisite to clearly potentiate chemotherapy-mediated antitumor activities, even in chemotherapy nonresponsive tumor models. This hypothesis is further strengthened by recently published data, indicating therapeutic synergy of cetuximab with BMS-275183, an oral taxane, in the cetuximab-sensitive L2987 and GEO tumor models (19). Moreover, others have reported synergy of cetuximab with drugs, such as gemcitabine, paclitaxel, docetaxel, topotecan, 5-fluorouracil, doxorubicin, and radiation (7, 9, 14, 15, 17, 18, 20, 31, 46, 47). The tumor models used in these studies were all at least borderline responsive to cetuximab monotherapy. Prewett et al. (16) previously showed enhanced antitumor activity of cetuximab in combination with CPT-11 in the HT29 human colon carcinoma xenograft, which stands in contrast to our observation in the same tumor model. However, in their hands, HT29 tumor growth was inhibited significantly more effectively by
Cetuximab monotherapy than we report, achieving tumor growth inhibitions in excess of 50% for at least one continuous TVDT. As such, HT29 would be classified at the minimum as a borderline-sensitive tumor model, thereby perhaps explaining the ability to potentiate antitumor activity of the chemotherapeutic agent.

In summary, cetuximab showed strikingly similar activity profiles in preclinical models of EGFR-positive tumors compared with the clinical setting. The identification of accurate biomarkers predicting the response to cetuximab should be aggressively pursued as they may not only significantly improve cetuximab monotherapy response rates but could also positively affect the success rate in treating patients in combination with various cytotoxic agents and/or radiation. Pharmacogenomic and proteomic approaches may be suitable to identify a set of genes or proteins, which will accurately reflect active and intact signaling through the EGFR and correlate with responsiveness to cetuximab treatment. Conversely, some markers may be discovered that predict resistance to the antibody therapy. Most recently, attempts were made to address the latter alternative. More specifically, Skvortsov et al. showed that they were able to identify proteins potentially involved in resistance mechanisms against cetuximab therapy by selecting two colorectal cancer cell lines with a high expression of EGFR but a different sensitivity toward treatment with the antibody (48). By comparing the proteomic profile of these cell lines, significant differences in the expression of 14 proteins were detected, which may serve as markers predicting resistance to this antibody therapeutic. Likewise, attempts were recently made to identify biomarkers predictive of response to cetuximab by profiling cetuximab-sensitive and cetuximab-resistant tumor cell lines as well as in vivo tumor xenografts (49, 50). Using pharmacogenomic and proteomic approaches, several candidate genes and proteins were identified, which are currently being assessed for their predictive value in the clinical setting.

Acknowledgments

We thank Drs. Robert Kramer and Jack Hunt for their support and critical comments on the article.

References


Molecular Cancer Therapeutics

Cetuximab preclinical antitumor activity (monotherapy and combination based) is not predicted by relative total or activated epidermal growth factor receptor tumor expression levels


Mol Cancer Ther 2006;5:104-113.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/5/1/104

Cited articles
This article cites 48 articles, 27 of which you can access for free at:
http://mct.aacrjournals.org/content/5/1/104.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/5/1/104.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.