Synergistic Antitumor Effects of Combined Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor Receptor-2 Targeted Therapy

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Abstract

**Purpose:** Combination therapies that target the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) pathways, are being actively tested for the treatment of cancer. In evaluating combination strategies, the ideal combination would be one in which the treatments interact in a way that is synergistic with regard to antitumor effects. Here, we have evaluated the interaction between anti-EGFR antibody Erbitux (cetuximab) and anti-VEGFR2 antibody, DC101, in preclinical models of pancreatic (BxPC-3) and colon (GEO) cancer.

**Experimental Design:** Analysis of the interaction between cetuximab and DC101 in vivo used a novel method for establishing the upper 95% confidence limits for the combination index (CI) of isobologram analyses, where CI < 1 indicates synergy. Assessment of tumor cell proliferation, apoptosis, VEGF production, and hypoxia, as well as tumor vascularization, was performed to gain insights into the mechanistic basis for synergy between agents targeting different tumor compartments.

**Results:** Monotherapy ED50 values for tumor growth inhibition ranged from 1.8 to 2.3 mg/kg and 10.5 to 16.6 mg/kg for cetuximab and DC101, respectively. From the dose response of the combination treatment, it was determined that cetuximab and DC101 are synergistic in the BxPC-3 (CI = 0.1, P < 0.01) and GEO (CI = 0.1, P < 0.01) models. Overlapping effects on the tumor cell and vascular compartments form a basis for the interaction, with VEGF production and hypoxia-inducible factor 1 potentially acting as molecular links between EGFR and VEGFR2 inhibition.

**Conclusions:** Results show antitumor synergy for combined EGFR and VEGFR2 targeted therapy, supporting the significant therapeutic potential of this combination strategy.
inhibitors of EGFR and VEGF are already showing promise in non–small cell lung cancer (16) and colorectal cancer (17).

Preclinical studies in cancer models are providing further support for this approach. In a s.c. GEO colon cancer cell xenograft model, increased tumor growth inhibition was observed with the combination of an antibody inhibitor of EGFR, cetuximab, and a VEGF antisense oligonucleotide, compared with the monotherapies (18). Furthermore, in an i.p. KM12L4 cell line model for colon cancer carcinomatosis (19) and an orthotopic gastric carcinoma model (11), cetuximab combined with a rat antibody to mouse VEGFR2 (DC101) inhibited tumor growth in excess of the effects of either monotherapy. Increased inhibition of tumor growth in mice treated with the combination of inhibitors of EGFR and VEGF signaling were associated with augmented reductions in microvascular density compared with treatment with an inhibitor of VEGF signaling when evaluated at the end of efficacy studies (11, 18). Consistent reduction in proliferation markers (e.g., Ki-67 and proliferating cell nuclear antigen) and increases in tumor cell and endothelial cell apoptosis (11, 18, 19) have also been reported in the combination group compared with the monotherapy dose groups that make up the combination.

Whereas these studies have shown that increased efficacy can be obtained by combining select doses of two monotherapies, the study designs used do not allow the evaluation of whether the two therapies interact in a synergistic manner, i.e., whether the effects of the combination are greater than expected from the potency of the monotherapies. The evaluation of synergism, absence of interaction, or antagonism between the two agents is a necessary step in fully understanding the therapeutic potential of the combination, and the interaction between inhibition of the EGFR and VEGFR signaling pathways (20). However, this has not been evaluated for EGFR and VEGF antagonists. A synergistic response between inhibitors of these two pathways would indicate an increased potential for inhibiting tumor cell growth or killing tumor cells, beyond that expected from the known effects of the individual monotherapies. Moreover, if a particular targeted therapy is associated with toxicities in patients, then it may be possible to combine two synergistic targeted agents at dose levels that reduce toxic side effects while maintaining the therapeutic benefit.

The interaction between agents is frequently determined in vitro using a median effect or isobologram analysis. However, analysis of drug interactions in vitro is not appropriate for testing the interaction between EGFR and VEGFR pathway inhibitors because in vitro assays cannot recapitulate the in vivo setting. Therefore, it is necessary to test these combinations of targeted agents in vivo to obtain a true measure of drug interaction. Testing for synergism in vivo cannot be achieved with the typical 2 × 2 design (control, monotherapy A, monotherapy B, A + B) frequently used to claim the benefits of a combination strategy. In fact, without additional dose response data, a study of this design can only be used to conclusively establish antagonism when the combination treatment is less efficacious than one or more of its constituents (20).

The present study uses an experimental design in which in vivo dose response data is obtained for cetuximab and DC101 monotherapy and for the combination, in human pancreatic BxPC-3 and colon GEO tumor xenograft models. The ratio of each monotherapy dose in the combination was kept fixed (21).

These three dose response relationships are then used to calculate a combination index (CI) that is used to discriminate between synergism and the absence of interaction. Upper confidence limits for CI are generated to determine whether synergism (CI < 1) exists with statistical confidence. We also performed mechanistic studies to show that the antitumor effects of therapies directed to the tumor cell compartment (cetuximab) or vascular compartment (DC101) of a tumor extended beyond the targeted compartment in the BxPC-3 and GEO models. In this way, the basis for an interaction between these two agents was established in the models used to establish synergy.

### Materials and Methods

**Cell lines.** The human pancreatic adenocarcinoma line BxPC-3 was obtained from the American Type Culture Collection (Manassas, VA). GEO human colon cancer cells were kindly provided by Dr. Lee Ellis (Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas). Cells were cultured at 37°C/5% CO2 in RPMI 1640 and McCoy’s 5A (In vitromotion Corporation, Carlbad, CA), respectively, supplemented with 10% fetal bovine serum (HyClone, Lenexa, KY), and 1% GlutaMAX (Invitrogen Corporation). Cells were passaged or collected for injection using Trypsin EDTA (Invitrogen Corporation).

**VEGF production ± cobalt chloride treatment.** Cells were grown to 80% confluence in complete medium and then grown overnight in serum-free media. Cells were continued in serum-free medium and cultured for 24 hours with either 20 μg/mL human IgG, human IgG + 100 μmo/L cobalt chloride (CoCl2; Sigma-Aldrich, St. Louis, MO), 20 μg/mL cetuximab, or cetuximab + CoCl2. Culture supernatants were then collected and cells were lysed with radioimmunoprecipitation assay buffer (Santa Cruz Biotechnolgy, Santa Cruz, CA). VEGF concentration was measured in supernatants with a human VEGF ELISA kit (R&D Systems, Minneapolis, MN). Protein concentration in cell lysates was measured using bicinchoninic acid (Pierce, Rockford, IL). Lysate protein (40 μg/lane) was run on a 4% to 12% NuPAGE gel (Invitrogen Corporation), transferred to nitrocellulose, and stained for hypoxia-inducible factor 1α (HIF-1α; mouse antibody from BD Biosciences, Franklin Lakes, NJ) or β-actin (Sigma-Aldrich). Enhanced chemiluminescence (Amersham, Piscataway, NJ) was used to develop images on X-ray film (Kodak, Rochester, NY).

**Xenograft models.** Experiments involving mice were done in accordance with protocols approved by the ImClone Systems Incorporated Internal Animal Care and Use Committee, and in accordance with current regulations and standards of the I.S. Department of Agriculture and the NIH. Six- to 7-week-old female athymic (nu/nu) mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for 1 week upon delivery. Each mouse was injected i.p. with 3 × 106 BxPC-3 cells or 10 × 106 GEO cells in 50% culture medium and 50% Matrigel (BD Biosciences, San Jose, CA). Tumor volumes were calculated as \( V = \frac{d_1 \times d_2 \times d_3}{2} \), where \( d \) is the longest diameter measured with calipers, and \( W \) is the diameter perpendicular to \( d \). When the mean tumor volume reached 250 to 350 mm3, mice were randomized by tumor volume into treatment groups and tumor volume was recorded twice weekly thereafter. Individual mouse tumor (%T/C) was calculated for each mouse as the relative tumor volume (individual mouse final tumor volume divided by initial tumor volume) divided by the relative tumor volume for the control group means, multiplied by 100. Partial tumor regression was defined as a final tumor volume less than the tumor volume measured on the first day of treatment.

**Treatment.** Cetuximab (20 mg/kg), a human IgG1 monoclonal antibody to human EGFR, and DC101, a rat monoclonal antibody to mouse VEGFR2/KDR, were produced at ImClone Systems Incorporated (Branchburg, NJ). Human IgG and rat IgG (Equitech-Bio, Inc., Kerrville, TX) were used as controls. All antibodies were diluted in USP saline.
The DC101/cetuximab fixed dose ratios in the combination group were evaluated on 6-overnight, and then embedded in paraffin. Hypoxyprobe binding was Sections were double-stained for HIF-1 mendations, using an FITC-conjugated antibody to hypoxyprobe. bufferged formalin at 4 by cervical dislocation. Tumors were harvested, fixed in 10% neutral (hypoxyprobe; Chemicon, Temecula, CA), 60 minutes prior to sacrifice treatment, mice were treated i.v. with 80 mg/kg hypoxyprobe-1 mg/kg cetuximab (Monday-Thursday), or the combination for 7 days. USP saline, 40 mg/kg DC101 (Monday-Wednesday-Friday), 5 or 10 included an IgG control [human IgG (5 mg/kg for BxPC-3 or 10 mg/kg for GEO, Tuesday-Friday) plus rat IgG (40 mg/kg, Monday-Wednesday-Friday)] and/or a USP saline control (10 μL/g, Monday-Wednesday-Friday). To evaluate the mechanism of action (see below) in the BxPC-3 and GEO xenograft models, five to six mice per group were dosed with USP saline, 40 mg/kg DC101 (Monday-Wednesday-Friday), 5 or 10 mg/kg cetuximab (Monday-Thursday), or the combination for 7 days. Histology. For hypoxyprobe studies, 7 days after the start of treatment, mice were treated i.v. with 80 mg/kg hypoxyprobe-1 (hypoxyprobe; Chemicon, Temecula, CA), 60 minutes prior to sacrifice by cervical dislocation. Tumors were harvested, fixed in 10% neutral buffered formalin at 4°C for 24 hours followed by 30% sucrose overnight, and then embedded in paraffin. Hypoxyprobe binding was evaluated on 6-μm sections according to the manufacturer’s recommendations, using an FITC-conjugated antibody to hypoxyprobe. Sections were double-stained for HIF-1α (Santa Cruz Biotechnology), visualized with streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA). Low-magnification digital images (×4) were acquired with a Nikon C1 confocal microscope (Nikon Instruments, Melville, NY) and the percentage of FITC-labeled viable tumor cell area was quantitated in one field of view for n = 5 to 6 tumors per group. In all other histology studies, tumors were removed 7 days after the start of treatment, fixed in 10% neutral buffered formalin at 4°C for 24 hours followed by 30% sucrose overnight, and then embedded in paraffin. Six-micrometer sections were stained for routine H&E, as well as the following immunohistochemistry markers using standard immunohistochemical techniques and 3,3′-diaminobenzadine as chromagen: activated (cleaved) caspase-3 (Cell Signaling Technology, Beverly, MA), Ki-67 (Lab Vision Corporation, Fremont, CA), phosphohistone H3 (pHistone-3; Upstate, Waltham, MA), and MECA-32 (BD Bioscience, San Diego, CA). Computer-assisted morphometric analysis of digital images was done using the Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). For the percentage of pyknotic/caspase-3-positive area, activated caspase-3 immunoreactive tumor areas containing a high frequency of small hematoxylin-positive pyknotic nuclei were measured in two H&E-stained tumor sections per mouse and expressed as a percentage of the viable tumor cross-sectional area. For quantification of the Ki-67 and pHistone-3-stained sections, images were segmented based on density and size thresholding to distinguish negative from positive objects, and cells were counted. For quantification of vessel density, MECA-32-positive vessels were counted manually on digital images. For all immunohistochemical quantifications, five randomly selected images each (total area, 7.3 mm²) from the tumor periphery and tumor core were analyzed. Tumor periphery indicates a region less than one field of view from the tumor edge at ×200 magnification (<1,360 microns), and tumor core indicates areas >1,360 microns from the tumor edge.

**VEGF production in vivo.** Seven days after the start of treatment (10 mg/kg cetuximab or USP saline, twice/wk), tumors were harvested and frozen in liquid nitrogen. Tumors were homogenized in 3 mL radioimmunoprecipitation assay buffer per gram of frozen tissue with a Polytron (Brinkmann Instruments, Westbury, NY). Protein concentration was measured in tumor homogenates using bichinonic acid (Pierce). VEGF was measured in tumor homogenates using a human VEGF ELISA kit (R&D Systems).

**Statistics.** For both monotherapy groups and the combination group, linear regression of the natural log (ln) of the total antibody dose versus individual mouse T/C% was used to provide regression coefficients for the intercept (x) and slope (β) of the regression line, as well as SEs for the regression coefficients for intercept (SEx) and slope (SEβ). For each of the three experimental treatment categories, a total of n = 31 to 35 mice were dosed in the BxPC-3 model and n = 24 to 30 mice were dosed in the GEO model. Letting A = ED50 for DC101, B = ED50 for cetuximab, and A + B = ED50 for the combination (A/B dose ratio in combination = k), the combination index = CI(A′, B′; A, B) = A/A′ + B/B′ ≤ 1 [or ln(CI) < 0] if the two treatments are synergistic (20). To determine an upper 95% confidence limit for CI, the variance of ln(CI) was determined as a function of k, A′/B′, and (A + B), and the values of β, SEx, and SEβ from each of the three regression analyses. Variance of ln(CI) was then used to determine if ln(CI) < 0 with statistical confidence. A detailed derivation of the variance of ln(CI) is provided in the Supplementary Data. The one-sided upper 95% confidence limit for ln(CI) = ln[CI(A′, B′; A, B)] + Z0.95 × var ln(CI)1/2, where Z0.95 = 1.65. For a 99% confidence limit Z0.99 = 2.33 was used. Whenever 0.0 is not in the confidence interval, CI is considered significantly <1.0 at the indicated confidence levels.

The frequency of regressions was analyzed using a χ² test. ELISA measurements and percentage of pyknotic area were compared by Kruskal-Wallis one-way ANOVA on ranks, followed by a Student-Newman-Keuls post hoc test (Sigma Stat; Systat Software, Inc., Point Richmond, CA). Unless otherwise indicated, all other histologic measurements were compared by two-way ANOVA on ranks, with treatment and tumor location (periphery or core) as factors, followed by Fisher’s partial least-squares difference post hoc test. The number of samples in each group was always taken as the number of mice. For all tests, P < 0.05 was considered significant.

### Results

Cetuximab and DC101 antibody treatments were tested independently and in combination at a range of doses to characterize the interaction between the treatments with regard to tumor growth inhibition (Table 1). In the BxPC-3 model, tumor growth was evaluated for 33 days of treatment, initiated when mean tumor volumes reached ~250 mm³ (Fig. 1).

<table>
<thead>
<tr>
<th>Model</th>
<th>DC101 Dose range (mg/kg)</th>
<th>Cetuximab Dose range (mg/kg)</th>
<th>Combination Dose range (mg/kg, DC101/Cetuximab)</th>
<th>ED50 (mg/kg)</th>
<th>ED50 (mg/kg)</th>
<th>ED50 (mg/kg)</th>
<th>CI (95% upper limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BxPC-3</td>
<td>0.4-40</td>
<td>16.6</td>
<td>0.05-5</td>
<td>1.8</td>
<td>0.4/0.05-40/5</td>
<td>0.86</td>
<td>0.1 (0.41)</td>
</tr>
<tr>
<td>GEO</td>
<td>0.4-40</td>
<td>10.5</td>
<td>0.1-10</td>
<td>2.3</td>
<td>0.4/0.1-40/10</td>
<td>0.74</td>
<td>0.12 (0.53)</td>
</tr>
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</table>

Table 1. Synergism analysis in BxPC-3 and GEO xenograft models
Because the saline and IgG control groups did not differ in terms of tumor growth, these two groups were pooled into a single control group for the calculation of individual mouse T/C% in the experimental groups. In both monotherapy and combination groups, a significant correlation was found between the ln(dose) and individual mouse T/C% (P < 0.02 for all three). ED50 values were 16.6 mg/kg for DC101 and 1.8 mg/kg for cetuximab (Table 1). For the combination group, the ED50 was 0.86 mg/kg, indicating a dose of 0.10 mg/kg cetuximab and 0.76 mg/kg DC101. These values were used to calculate CI [(0.1/1.8) + (0.76/16.6) = 0.1], indicating synergism.

Although a calculated CI < 1 could support synergism, the statistical confidence for this conclusion needs to be determined. The method presented here was developed to estimate the confidence limits for CI directly to evaluate the degree of synergism (see Materials and Methods). This method estimates the variance for ln(CI) to determine whether ln(CI) < 0 with 95% confidence, with the assumption that if ln(CI) were repeatedly measured, the values would be normally distributed. The estimated variance is a function of common variables derived from a linear regression analysis used by numerous statistical software programs. In the BxPC-3 model, the estimated CI is 0.1 for a combination of cetuximab and DC101, and the upper 95% and 99% confidence limits for CI are 0.41 and 0.74, respectively, demonstrating that synergism exists between these two treatments in the BxPC-3 pancreatic cancer model. Further support for the increased antitumor potential of the combination therapy comes from the finding that at the highest combination doses, 10/1.25 and 40/5 mg/kg of DC101/cetuximab, 80% and 40% of tumors showed partial tumor regression, respectively, compared with 0% of mice in both control groups and <20% in all monotherapy groups (P = 0.0005).

To show that the synergism between VEGFR2 and EGFR inhibition was not limited to the BxPC-3 pancreatic cancer model, the combination of cetuximab and DC101 was also tested in the GEO colon cancer model. In this study, the DC101/cetuximab dose ratio in the combination group was lowered to 4:1. Because the saline and IgG control groups did not differ in terms of tumor growth (Fig. 2), these two groups were pooled into a single control group for the calculation of individual mouse T/C% in the experimental groups. In both monotherapy and combination groups, a significant correlation was found between the ln(dose) and individual mouse T/C% (P < 0.02 for all three). ED50 values were 10.5, 2.3, and 2.0 mg/kg DC101.
0.74 mg/kg for DC101, cetuximab, and the combination groups, respectively (Table 1). CI for this model was 0.12 with 95% and 99% upper confidence limits for CI of 0.53 and 0.97, respectively, demonstrating synergism between DC101 and cetuximab in the GEO colon cancer model. As in the BxPC-3 model, there was a treatment-related increase in the frequency of partial tumor regressions (P = 0.004). In the high-dose combination group, 100% of mice showed regression, compared with 60% and 50% in the high-dose DC101 and cetuximab monotherapy groups, respectively, and 11% in the pooled control group.

Synergism between treatments can be the result of an interaction between the downstream effects of each monotherapy, or a more upstream interaction between the molecular pathways affected by each monotherapy. To gain an understanding of the potential downstream cellular and vascular effects contributing towards the synergy between cetuximab and DC101, BxPC-3 and GEO tumor xenografts were analyzed histologically for effects on tumor cell apoptosis and proliferation, as well as effects on tumor vascularization. In order to maximize the sensitivity in detecting the effects that could contribute towards the antitumor activity of DC101 and cetuximab, the doses selected for this analysis were the highest doses used in the isobologram studies above (two to five times the ED$_{50}$ values). The combination group in this analysis was used to test for potential antitumor effects that were clearly increased compared with the monotherapy groups, as an indicator that this effect could be part of the mechanism for synergy. However, it is important to note that a lack of increased effect in the combination group compared with monotherapy groups at the relatively high dose levels used (two to five times the ED$_{50}$ values) does not exclude a contribution towards synergy at lower effect levels. Conclusive determination of synergy would require isobologram studies similar to those reported above, with quantitation of mechanistic markers instead of tumor volume.

To minimize factors such as tumor size, inflammation, and necrosis that could complicate data interpretation when samples are evaluated at the end of efficacy studies, we evaluated tumors after 7 days of therapy. At this time point, the tumor growth curves were just beginning to separate (Figs. 1 and 2) and inhibitors of VEGFR (22) and EGFR (23) signaling were already reported to reduce tumor microvessel density.

BxPC-3 cells were observed in hematoxylin-stained slides to grow in clusters of tumor cells, often surrounding duct-like structures (Fig. 3A). Tumor cell clusters were surrounded by fibrotic eosin-stained connective tissue. H&E-stained tumor sections were very similar in all treatment groups with regard to fibrosis, area of duct-like structures, and the percentage of the tumor area with a high density of tumor cells (quantification not shown). In spite of general histologic similarity, distinct regions of focal clusters of activated caspase-3-positive pyknotic tumor cells were observed in zero of six cetuximab, three of six DC101, and six of six DC101 + cetuximab–treated tumors (Fig. 3B and C). The percentage of total tumor area represented by these regions was quantified in H&E-stained sections. Although DC101 and cetuximab did not significantly increase the percentage of pyknotic areas compared with controls, a significant increase in these regions was observed when cetuximab therapy was added to DC101 (Fig. 3D). This increase in the combination group, although only representing ~ 10% of the viable tumor cell area, was statistically significant compared with all other treatment groups, including DC101 and cetuximab monotherapies (P < 0.05). Negative staining controls, including Ki-67 (isotype-matched primary antibody control), did not stain the pyknotic regions discussed above (data not shown), supporting the conclusion that activated caspase-3 staining was specific. Thus, an increase in regional tumor cell apoptosis is considered to be part of the mechanism for synergy between cetuximab and DC101 in the BxPC-3 model.

In hematoxylin-stained slides, GEO cells were observed growing in clusters of tumor cells, although these clusters tended to be more connected than in BxPC-3 (Fig. 3E). In addition, control GEO tumors exhibited a higher percentage of necrotic tumor regions (data not shown). As in BxPC-3, tumor regions rich in activated caspase-3-positive, pyknotic tumor cells were observed in GEO tumors (Fig. 3F and G). As a percentage of viable, nonnecrotic tumor areas, these regions were significantly increased in DC101 and combination-treated tumors (Fig. 3H), to the point where 40% to 45% of viable tumor regions were undergoing apoptosis after 7 days of treatment (P < 0.001). There was no significant difference between the effect of DC101 and the combination. With regard to the mechanism for synergy between cetuximab and DC101, in GEO, the benefit of adding cetuximab to DC101 for increased tumor cell apoptosis may be dampened or difficult to detect given the dramatic effect of DC101 alone in the tumor cell compartment. Therefore, this analysis did not provide evidence for the involvement of tumor cell apoptosis in the mechanism for synergy in the GEO model, although as mentioned above, this does not exclude a contribution towards synergism at lower dose and effect levels.

Immunohistochemical markers of proliferation and blood vessels in BxPC-3 tumors were found to differ between the tumor periphery and core, therefore, these regions were analyzed separately (Fig. 4). Ki-67 was used as a marker for cells that were not in the resting G0 state, as a measure of proliferative potential. The percentage of Ki-67-positive cells in BxPC-3 tumors was higher in the periphery than in the core (Fig. 4A). Cetuximab significantly reduced the percentage of Ki-67-positive cells (P < 0.03), although only in the tumor periphery. DC101 did not affect the percentage of Ki-67-positive cells in either compartment. Combining cetuximab with DC101 resulted in a significant reduction in the percentage of Ki-67-positive cells in both compartments (P < 0.02). The percentage of tumor cells positive for pHistone-3, a marker of cells undergoing mitosis, was low (0.5-1.5% of tumor cells) in this xenograft model, and tended to be more prevalent in the tumor periphery (Fig. 4B). Interestingly, cetuximab did not affect the percentage of tumor cells positive for pHistone-3 after 7 days of treatment, although DC101 caused a significant reduction in the percentage of tumor cells positive for pHistone-3 in the tumor periphery (P < 0.03). The combination of DC101 and cetuximab had a very similar effect to DC101 monotherapy. Although the effects of combination treatment on proliferation markers were more consistent than either monotherapy, the effect of the combination was similar to either DC101 or cetuximab monotherapy.

In GEO tumors, the difference between the tumor core and periphery was less than in BxPC-3. The percentage of Ki-67-positive cells was similar in the tumor periphery and the tumor.
core in GEO (Fig. 4D). Cetuximab and the combination of cetuximab and DC101, significantly reduced the percentage of Ki-67-positive cells \( (P < 0.001; \text{Fig. 4D}) \). DC101 also reduced the percentage of Ki-67-positive cells \( (P = 0.01) \), but to a lesser extent than the cetuximab-containing treatment regimens (Fig. 4D). Notably, only the combination treatment group significantly reduced the percentage of tumor cells positive for pHistone-3 (Fig. 4E). The difference between the combination and monotherapy groups was statistically significant \( (P < 0.02) \), indicating that reduced tumor cell proliferation may be part of the mechanism for synergy between the two agents in the GEO model.

With respect to the effects of tumor vascularization in the BxPC-3 model, DC101 and cetuximab + DC101 significantly reduced the number of MECA-32-positive blood vessels per unit of tumor area in both the tumor core and tumor periphery after...
7 days of treatment ($P < 0.001$; Fig. 4C). Cetuximab also reduced blood vessel density ($P < 0.05$). Differences in tumor vascularity between the combination cetuximab + DC101 group, and monotherapy groups did not reach statistical significance ($P > 0.16$) indicating that cetuximab does not augment the maximal antivascular effects of VEGFR2 inhibition with DC101 in this model. Similarly in the GEO model, DC101 and the combination groups showed similar and significant reductions in microvessel density ($P < 0.03$; Fig. 4F). Cetuximab only reduced blood vessel density in the tumor periphery in the GEO model ($P = 0.012$ by one-way ANOVA on ranks followed by Fisher’s least-squares difference post hoc test).

The effects of cetuximab and DC101 monotherapy, as expected, focuses on the tumor cellular and vascular compartments, respectively. However, it is also clear that the effects of these agents could extend into the other compartment, establishing a general basis for an interaction. Effects in the combination group indicate that this interaction results in reduced tumor cell proliferation in GEO and increased regional apoptosis in BxPC-3, contributing towards synergy between cetuximab and DC101. Because these effects in the tumor cellular compartment were observed at high antibody doses, they may also contribute towards the increase in tumor regressions reported above. At lower doses, other interactions, including interactions between the overlapping effects of DC101 and cetuximab in the vascular compartment, may be part of establishing synergism.

Interactions between effects in the same tumor compartment may occur without overlap in the molecular pathways affected by two synergistic treatments. However, in this case, cross-talk between the EGFR and VEGFR2 pathways has been shown, indicating that molecular pathways may exist for establishing a synergistic interaction between cetuximab and DC101. In particular, cetuximab treatment of A431 human epidermoid carcinoma cells reduces VEGF production (23). VEGF was therefore evaluated in the BxPC-3 and GEO cell lines to

![Fig. 4. Treatment effects on tumor proliferation markers and vascularization. A-C, BxPC-3 xenografts; D-F, GEO xenografts. Percentage of Ki-67- (A and D) and pHistone-3- (B and E) positive tumor cells, and the density of MECA-32-positive blood vessels (C and F) in the tumor periphery (black columns) and core (open columns), after 7 days of treatment (columns, means bars, ± SE; n = 5–6 per group). Treatments include 40 mg/kg DC101, twice/wk; 5 mg/kg cetuximab for BxPC-3, 10 mg/kg for GEO, twice/wk. Insets, typical staining patterns in saline-treated control tumors. Calibration bars, 100 μmol/L.]
determine if the same potential for interaction between EGFR and VEGFR2 pathways exists in the models used to establish synergism.

BxPC-3 and GEO cells produced VEGF in vitro and VEGF production was increased with the HIF-1α stabilizing agent CoCl2 (24). EGFR inhibition with cetuximab reduced VEGF output into the supernatant in normal or CoCl2-stimulated conditions in both cell lines (Fig. 5A). The link between the VEGF/VEGFR2 pathway and the EGFR pathway through VEGF is also functional in vivo, where 7 days of treatment with cetuximab caused a dramatic inhibition of the concentration of VEGF produced by tumor cells in BxPC-3 and GEO tumors (P < 0.0001; Fig. 5B). These results show a potential molecular basis in the BxPC-3 and GEO models for establishing a synergistic interaction between cetuximab and DC101 in the vascular compartment.

Similar to the overlap in the VEGF pathway in the vascular compartment, the potential for overlap exists in the cellular compartment as well. The antivascular effects of DC101 can result in tumor cells existing in a more hypoxic environment (10). Stabilization of HIF-1α in hypoxic tumor cells results in increased transcription of hypoxia-regulated genes, such as VEGF, Glut1, and carbonic anhydrase-9, in an effort to increase angiogenesis, anaerobic metabolism, and lower the pH, respectively (25). Cetuximab can regulate HIF-1α levels, reducing HIF-1α in A431 cells with and without HIF-1α stabilization induced by deferoxamine (26). In BxPC-3 and GEO cells, cetuximab was shown to reduce the stability of HIF-1α during treatment with the HIF-1α stabilizing agent CoCl2 (Fig. 5C), in parallel with the reduction in CoCl2-induced VEGF release into the supernatant (Fig. 5A). The regulation of HIF-1α by cetuximab could be important in hypoxic conditions reportedly increased by DC101 treatment (10). We therefore tested for DC101-induced hypoxia in the BxPC-3 and GEO models.

In BxPC-3 xenografts, hypoxic tumor regions bound by pimonidazole hydrochloride (hypoxprobe) consisted of isolated islands of tumor cells (Fig. 6B and C). In GEO xenografts, hypoxprobe was concentrated in regions bordering necrotic areas (Fig. 6E and F). In BxPC-3 tumors, there was no change in the percentage of viable tumor areas stained with hypoxprobe after 7 days of DC101 treatment (Fig. 6A). In GEO tumors, however, DC101 significantly increased the hypoxic tumor cell area and staining intensity (Fig. 6D). One tumor in the salinetreated group was significantly hypoxic (13% of viable regions showing hypoxia), compared with the other four tumors in this group. This tumor was considered an outlier by a maximum normal residuals test (P < 0.01; ref. 27). Without this outlier, the effect of DC101 reached statistical significance in GEO tumors by Student’s t test (P = 0.03). In addition, setting a normal level of hypoxia at <2.5% (Fig. 6D), five of six DC101-treated tumors were hypoxic, compared with only one of five controls (P = 0.03; χ2 test).

Hypoxprobe binding, indicative of hypoxic conditions, overlapped significantly with HIF-1α immunoreactivity (Fig. 6), supporting the regulation of HIF-1α stability by hypoxia in GEO tumors. From this data, showing that DC101 induced hypoxia, as well as the in vitro data (Fig. 5C) and published literature (26, 28) indicating that HIF-1α and its proliferative/antiapoptotic effects can be regulated by EGFR inhibitors, HIF-1α represents a potential molecular pathway for establishing a synergistic interaction between cetuximab and DC101 in the tumor cellular compartment in the GEO model.

**Discussion**

Inhibition of EGFR or VEGFR signaling has shown antitumor efficacy in both preclinical models and in patients (see Introduction). Preclinical and clinical data support an increase in antitumor effect when combining an efficacious dose of an EGFR antagonist with a VEGFR antagonist (11, 16, 17, 19). However, these analyses were not designed to characterize the interaction between the pathways. In fact, results demonstrating an increase in efficacy with a combination of treatment A + treatment B, compared with either treatment A or treatment B alone, cannot discriminate between synergism and antagonism without more information on the dose response relationships. Establishing synergism between two treatments with respect to their antitumor effects could have important implications for reducing treatment-related toxicities and increasing therapeutic...
benefit. Therefore, we have developed and used methods to test for synergism between antibody antagonists of EGFR (cetuximab) and VEGFR2 (DC101) in vivo. Results show a significant synergism between these two pathways with respect to tumor growth inhibition in xenograft models of both pancreatic and colon cancers.

The method developed and used for establishing synergism between two treatments derives from isobologram analysis which makes use of a combination index, discussed in detail by Berenbaum (20). In this analysis, CI = 1 indicates the absence of interaction between inhibitors of the two pathways and CI < 1 indicates synergy. By deriving a variance for ln(CI) (see Supplementary Data) we were able to establish statistical confidence in the conclusion that inhibition of EGFR and VEGFR2 pathways is synergistic, and provide upper limits for CI. An alternative method exists that uses the same experimental design, but instead of focusing on CI, the measured ED50 and confidence limits for the combination group are compared with the combination group ED50 predicted from the monotherapy ED50 values and confidence limits, assuming the absence of interaction (21). In the present study, we focused on establishing limits for CI because this is the main variable used in the literature to evaluate the interaction between treatments. CI, and its limits, could then be used to establish synergy and obtain an estimate of its magnitude. To accomplish this, it is noted that CI is actually the sum of two fractions (20), that when multiplied by 100, represents the percentage of each monotherapy ED50 in the combination treatment which results in an individual mouse T/C% = 50. When there is no interaction between the treatments, these two percentages will add up to 100% (CI = 1), but when CI = 0.1, they add up to only 10%. The finding that CI = 0.1 for both the BxPC-3 and GEO models indicates that, on average, only 10% (41% and 53%, upper 95% confidence limits, respectively) of the expected total dosages of the efficacious monotherapies are necessary to achieve the targeted efficacy level, when the treatments are given in combination. The potency of the combination treatment was also supported by the increased frequency of partial tumor regressions in the high-dose combination groups.

Given the known effects of EGFR inhibitors and VEGFR inhibitors on tumor cell proliferation, tumor cell survival and tumor vascularization, we began our efforts to establish a mechanistic basis for an interaction with these variables. DC101 caused a dramatic antivasculary effect, reducing tumor microvessel density in BxPC-3 and GEO models. The antivasculary effects of DC101 on blood vessel density have previously been observed (10), and are thought to play a significant role in the tumor growth–inhibitory effects of this antibody. Cetuximab significantly reduced the percentage of tumor cells expressing Ki-67 in both models, as previously described (11, 18, 19). The effects on this marker of proliferative potential in two models in which cetuximab significantly inhibits tumor growth is in line with correlations between Ki-67 reduction and clinical benefit following EGFR inhibition in patients (29).

The vascular and cellular effects of DC101 and cetuximab support the targeting of receptors on cells in the vascular and cellular tumor compartments, respectively. However, it is also clear that the antitumor effects of cetuximab can be transmitted to the vascular compartment. This is accomplished on a molecular level through a significant reduction in VEGF concentration in tumors in response to cetuximab, as previously shown in A431 cells (23) and GEO cells (18). Because microvessel density depletion was similar in tumors treated with relatively high doses of DC101 monotherapy and combination-treated mice, the interaction between the effects of DC101 and cetuximab in the vascular compartment does not seem to increase maximal antivasculary effects, but may still contribute to synergy at lower effect levels. This could occur, for example, if lowering of tumor VEGF levels significantly increased the ability of DC101 to compete with VEGF for binding to VEGFR2.
Isobologram studies with, for example, microvessel density as the measured effect, are necessary to show this conclusively.

In the present study, the lack of increased microvessel depletion with combination therapy, compared with DC101 monotherapy, differs from the results reported in another model. In a KM12L4 human colon cancer carcinomatosis model, DC101 in combination with cetuximab reduced microvessel density compared with DC101 alone (11). Notably, this study treated mice with 0.8 mg/dose DC101 every 3 days, compared with the more intense 40 mg/kg DC101 (~1 mg/dose, Monday-Wednesday-Friday dosing regimen) used in the present study. However, in a TMK-1 gastric cancer xenograft model (19), cetuximab did not increase the reduction in tumor vascularity observed with DC101 at 0.8 mg/dose every 3 days. Thus, the benefit of adding cetuximab to DC101 towards increasing the maximal reduction in microvessel density may be model-dependent. Because reduction of VEGF by cetuximab could affect not only the VEGF-R2 pathway blocked by DC101, but could reduce signaling through VEGFR1 (15), the evaluation of the role of this pathway in tumor angiogenesis may prove to be important towards understanding this model dependence.

The effects of cetuximab in the vascular compartment establishes a basis for interaction between cetuximab and DC101 in this compartment, but the effects of DC101 also extend beyond the initially targeted vascular compartment. DC101 had significant effects on tumor cells, including increased regional tumor cell apoptosis in GEO tumors and reduced expression of proliferation markers in BxPC-3 and GEO tumors. The more dramatic apoptotic effects of DC101 in GEO tumors may be related to a better ability to reduce the tumor cell blood supply, indicated by increased hypoxyprobe binding. Increased apoptosis of tumor cells has been noted by terminal nucleotidyl transferase-mediated nick end labeling staining following treatment with DC101 when evaluated at the end of efficacy studies (11, 19), although the focal regional apoptosis noted in the present study early in the course of treatment has not been reported. Interestingly, similar to results with pHistone-3 in the BxPC-3 model, tumor cell proliferation evaluated with proliferating cell nuclear antigen as the marker was not affected by cetuximab but was reduced by DC101 in a colon cancer carcinomatosis model (11).

Based on effects detected only in the DC101 + cetuximab combination group, the end result of the interaction between the tumor cellular effects of DC101 and cetuximab was an increased regional tumor cell apoptosis in BxPC-3 tumors. In GEO tumors, this interaction resulted in reduced tumor cell mitosis in the combination group. Because tumor growth is controlled through a balance between tumor cell proliferation and apoptosis (30), these cellular effects detected only in the combination group are thought to play a role in establishing synergy between cetuximab and DC101 in the BxPC-3 and GEO models with respect to tumor growth inhibition.

The interaction between EGFR and VEGFR2 inhibition in the cellular compartment causing the increased cellular effects in the combination group could occur at the HIF-1 signaling pathway. Increased HIF-1 transcriptional activity following HIF-1α stabilization can increase both apoptotic pathways and survival/proliferation pathways (25). Autocrine survival/proliferation pathways activated in tumor cells by HIF-1 include the transforming growth factor-α/EGFR pathway in renal carcinoma cells (28) and the VEGF/VEGFR1 pathway in the SK-N-RE(2) neuroblastoma cell line (31). Increased HIF-1 signaling in hypoxic conditions during DC101 treatment (10, 32) may therefore, to some extent, support the growth and survival of tumor cells, in addition to stimulating angiogenesis. Cetuximab has the potential to block the EGFR autocrine loop directly, as well as to reduce HIF-1 signaling in general through a reduction in HIF-1α stability (26). This may be particularly relevant in the GEO model in which DC101 increased hypoxyprobe binding. It is puzzling that in spite of a reduction in microvessels in BxPC-3 tumors following DC101 treatment, increased hypoxyprobe binding was not detected. This finding may be related to the vessel maturation effects of VEGF antagonists associated with improved oxygenation (33), and points to the potential difference in the mechanism for synergy between DC101 and cetuximab in different models.

To summarize, we have shown that specific and targeted inhibition of EGFR and VEGFR2 signaling are highly synergistic in both pancreatic (BxPC-3) and colon (GEO) tumor xenograft models. Although cetuximab and DC101 target EGFR and VEGFR2 in the tumor cellular or vascular compartment, respectively, the effects of cetuximab and DC101 extend into both compartments, establishing a general basis for an interaction in both compartments. A potential interaction between cetuximab and DC101 at the molecular level in the vascular compartment exists through the regulation and importance of tumor VEGF. In the cellular compartment cetuximab and DC101 may interact at the level of the HIF-1 pathway. Results suggest that the therapeutic benefit of dual inhibition of these pathways in patients may exceed that expected from the reported effects of EGFR or VEGFR inhibitors administered alone, and support the significant therapeutic potential of this combination strategy in the treatment of cancer.

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EGFR and VEGFR2 Antibody Synergy


Synergistic Antitumor Effects of Combined Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor Receptor-2 Targeted Therapy

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