Treatment of Human Tumor Xenografts with Monoclonal Antibody 806 in Combination with a Prototypical Epidermal Growth Factor Receptor – Specific Antibody Generates Enhanced Antitumor Activity

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Abstract
Monoclonal antibody (mAb) 806 is a novel epidermal growth factor receptor (EGFR) antibody with significant antitumor activity that recognizes a mutant EGFR commonly expressed in glioma known as delta2-7 EGFR (de2-7 EGFR or EGFRvIII) and a subset of the wild-type (wt) EGFR found in cells that overexpress the receptor. We have used two human xenograft mouse models to examine the efficacy of mAb 806 in combination with mAb 528, a prototypical anti-EGFR antibody with similar specificity to cetuximab. Treatment of nude mice, bearing s.c. or i.c. tumor human xenografts expressing the wt or de2-7 EGFR, with mAbs 806 and 528 in combination resulted in additive and in some cases synergistic, antitumor activity. Interestingly, mAb 528 was also effective against xenografts expressing the ligand independent de2-7 EGFR when used as a single agent, showing that its antitumor activity is not merely mediated through inhibition of ligand binding. When used as single agents, neither mAbs 806 or 528 induced down-regulation of the de2-7 EGFR either in vitro or in vivo. In contrast, the combination of antibodies produced a rapid and dramatic decrease in the total cell surface de2-7 EGFR both in vitro and in xenografts. Consistent with this decrease in total cell surface de2-7 EGFR, we observed up-regulation of the cell cycle inhibitor p27KIP1 and a decrease in tumor cell proliferation as measured by Ki-67 immunostaining when the antibodies were used in combination in vivo. Thus, mAb 806 can synergize with other EGFR-specific antibodies thereby providing a rationale for its translation into the clinic.

Overexpression of the EGFR has been observed in many epithelial tumors, with increased EGFR expression levels often correlating with a poorer clinical prognosis (1–3). This overexpression of the receptor is often caused by amplification of the EGFR gene (4). EGFR gene amplification and subsequent overexpression of the EGFR protein is particularly prevalent in gliomas, the most common primary tumor of the central nervous system (4). Indeed, the highly malignant glioblastoma multiforme exhibits EGFR gene amplification at a frequency of 40% to 50%, with many tumors also displaying structural rearrangements of the EGFR gene (4). The most common of these variant EGFR genes contains an in-frame 801-bp deletion that removes exons 2 to 7 (5, 6). This resulting receptor, known as the de2-7 EGFR, does not bind ligand but displays a low level of constitutive activity and imparts a significant in vivo growth advantage to a number of cell types including glioma (7–9). Glioma cells usually coexpress both the de2-7 and wild-type (wt) EGFR, with de2-7 EGFR expression generally associated with higher antitumor activity that recognizes a mutant EGFR commonly expressed in glioma known as delta2-7 EGFR (de2-7 EGFR or EGFRvIII) and a subset of the wild-type (wt) EGFR found in cells that overexpress the receptor. We have used two human xenograft mouse models to examine the efficacy of mAb 806 in combination with mAb 528, a prototypical anti-EGFR antibody with similar specificity to cetuximab. Treatment of nude mice, bearing s.c. or i.c. tumor human xenografts expressing the wt or de2-7 EGFR, with mAbs 806 and 528 in combination resulted in additive and in some cases synergistic, antitumor activity. Interestingly, mAb 528 was also effective against xenografts expressing the ligand independent de2-7 EGFR when used as a single agent, showing that its antitumor activity is not merely mediated through inhibition of ligand binding. When used as single agents, neither mAbs 806 or 528 induced down-regulation of the de2-7 EGFR either in vitro or in vivo. In contrast, the combination of antibodies produced a rapid and dramatic decrease in the total cell surface de2-7 EGFR both in vitro and in xenografts. Consistent with this decrease in total cell surface de2-7 EGFR, we observed up-regulation of the cell cycle inhibitor p27KIP1 and a decrease in tumor cell proliferation as measured by Ki-67 immunostaining when the antibodies were used in combination in vivo. Thus, mAb 806 can synergize with other EGFR-specific antibodies thereby providing a rationale for its translation into the clinic.

Inhibition of the EGFR is a rational strategy for the development of new cancer therapeutics (11). Potential therapeutics include anti-EGFR antibodies (12) and small molecular weight tyrosine kinase inhibitors of the EGFR (13). A number of antibodies directed to the extracellular domain of the EGFR have now been tested in the clinic including EMD 55900 (14), ABX-EGF (15), and IMC-C225 (16), all of which have displayed some antitumor activity in patients (12). The most clinically advanced of these is IMC-C225, which is currently being tested in phase II/III clinical trials for the treatment of head and neck, colorectal, and non–small cell lung carcinomas (17) and was recently approved for use in Europe. It has been presumed that the antitumor activity of these antibodies is primarily related to their ability to block ligand binding, but further antitumor mechanisms such as immune effector function, receptor down-regulation, induction of inappropriate signaling, and interference with receptor...
dimerization and/or oligomerization need to be considered in more detail. One limitation of antibodies targeting the wt EGFR is that they show significant uptake in normal tissue such as the liver and skin (18–20). Whereas targeting of normal tissues seems to cause manageable side effects such as skin rash (20), it does mean that these antibodies would generate significant side effects if coupled to cytotoxic agents or radioisotopes for antitumor therapy.

Monoclonal antibody (mAb) 806 is a novel anti-EGFR specific antibody that was generated from mice immunized with de2-7 EGFR-transfected NR6 fibroblasts (21, 22). Interestingly, whereas mAb 806 clearly binds the de2-7 EGFR, it also binds to a subset of the wt EGFR (~10%) expressed on the surface of A431 cells (a vulval carcinoma cell line that overexpresses the EGFR; ref. 22). Recent analysis shows that mAb 806 recognizes a transitional form of the EGFR before it becomes activated (23, 24). Binding of mAb 806 to this transitional form of the EGFR prevents receptor activation and subsequent signaling. Unlike the wt EGFR, the de2-7 EGFR is constitutively in this transitional conformation and thus available for mAb 806 binding. Our previous studies have shown that treatment of glioma xenografts that express the de2-7 EGFR with mAb 806 causes significant inhibition of tumor growth (25). Significantly, established A431 tumor xenografts were also inhibited by mAb 806 despite the antibody only recognizing a small proportion of the EGFR expressed in these cells (25).

Given the unique properties of mAb 806, we decided to investigate its efficacy in combination with mAb 528, a prototypical anti-EGFR antibody. The 528 antibody acts as a ligand antagonist and inhibits the growth of the C225 antibody and displays very similar properties (26). We investigated its efficacy in combination with mAb 528, a prototypical anti-EGFR antibody. The 528 antibody was shown to inhibit cephalosporin (Sigma Chemical Co., St. Louis, MO), following serum starvation for 36 hours. Cells were washed in ice-cold PBS before lysis in cold lysis buffer (30 mmol/L HEPES, 150 mmol/L NaCl, 10 mmol/L NaF, 1% Triton X-100, 200 mmol/L NaO,V, 0.4% H2O2, and 0.4% SDS) and the protease inhibitor cocktail set 1 (Calbiochem) containing 500 mmol/L AEBSF, 150 mmol/L aprotinin, 1 mmol/L E-64 protease inhibitor, 0.5 mmol/L EDTA, and 1 mmol/L leupeptin (pH 7.4)). Each volume of each sample was mixed with SDS sample buffer and loaded onto 12% Tris-gels to 12% NuPage gels (Invitrogen Life Technologies, Malagrove, Canada). Each lysis experiment and immunoblot was repeated at least thrice.

Preparation of xenograft homogenates. Xenograft homogenates of tumors excised from three different mice per treatment group were prepared by homogenization in immunoprecipitation assay buffer (50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 200 mmol/L NaO,V, 0.4% H2O2, 0.5% deoxycholate, 0.005% SDS, 10 mmol/L NaF, and the protease inhibitor cocktail set 1). Homogenization was conducted in a Dounce Homogeniser before incubation for 1 hour at 4°C. Preparations were subsequently clarified by centrifugation at 14,000 × g for 30 minutes at 4°C. The protein content of the resultant supernatants was determined using the detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer’s instructions. Samples were equalized for total protein, mixed in an equal volume of SDS sample buffer and loaded onto 4% to 12% NuPage Bis-Tris gels.

Immunoblotting. After transfer to polyvinylidene difluoride membranes, separated proteins were detected by immunoblotting with anti-EGFR (Cell Signaling Technology, Beverly, MA), anti-phospho-EGFR-1173 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-p27KIP1 antibodies. To ensure equal loading, immunoblots were probed with anti-α-tubulin antibody (Santa Cruz Biotechnology) and separate gels stained with Coomassie. Ki-67 immunostaining. Xenografts were removed, embedded in Tissue Tek ovenite cardamyl transferase compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at −80°C. Ten-micrometer sections were cut, fixed in acetone, and stained with sheep anti-human Ki-67 polyclonal antibody (Chemicon International, Temecula, CA) for 1 hour. After rinsing, a rabbit anti-sheep-heraldish peroxidase antibody (Chemicon International) was applied and bound antibody detected with AEC substrate solution (0.1 mol/l acetic acid, 0.1 mol/l sodium acetate, 0.02 mol/l AEC, and 0.03% H2O2). Slides were then counterstained in hematoxylin (BDH Laboratory, Poole, United Kingdom) and mounted. The percentage of reactive tumor nuclei to the Ki-67 antibody was estimated by examining an average of 17 representative high-power fields per treatment group containing a total of ~8,000 cells. Data were expressed as proliferation index (PI) using the formula: (cells positive for Ki-67 staining / total cells) × 100.

Immunohistochemical analysis of microvessel density. Xenografts were removed and fixed in 10% buffered formalin and used for

Materials and Methods

Cell lines and monoclonal antibodies. The de2-7 EGFR-transfected cell line, U87MG.D2-7 and the U87MG.DK cell line expressing a kinase inactive variant of the de2-7 EGFR, have been described previously (7, 28). The A431 cell line has also been described previously (22). The p27KIP1 (Santa Cruz Biotechnology) antibodies. To ensure equal loading, immunoblots were probed with anti-α-tubulin antibody (Santa Cruz Biotechnology) and separate gels stained with Coomassie. Ki-67 immunostaining. Xenografts were removed, embedded in Tissue Tek ovenite cardamyl transferase compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at −80°C. Ten-micrometer sections were cut, fixed in acetone, and stained with sheep anti-human Ki-67 polyclonal antibody (Chemicon International, Temecula, CA) for 1 hour. After rinsing, a rabbit anti-sheep-heraldish peroxidase antibody (Chemicon International) was applied and bound antibody detected with AEC substrate solution (0.1 mol/l acetic acid, 0.1 mol/l sodium acetate, 0.02 mol/l AEC, and 0.03% H2O2). Slides were then counterstained in hematoxylin (BDH Laboratory, Poole, United Kingdom) and mounted. The percentage of reactive tumor nuclei to the Ki-67 antibody was estimated by examining an average of 17 representative high-power fields per treatment group containing a total of ~8,000 cells. Data were expressed as proliferation index (PI) using the formula: (cells positive for Ki-67 staining / total cells) × 100.

Immunohistochemical analysis of microvessel density. Xenografts were removed and fixed in 10% buffered formalin and used for
microvessel density detection. Sections of 4-μm thickness were cut, mounted onto Superfrost Plus slides (Menzel-Glaser, Germany), and deparaffinized in a 60°C oven for 30 minutes followed by 2 × 5 minutes xylene rinses and 2 × 5 minutes 100% ethanol rinses. Sections were then rehydrated in distilled water and microwaved for 15 minutes in EDTA buffer (pH 8.0; Lab Vision Co., Fremont, CA) for antigen retrieval. Endogenous peroxidase was quenched before blocking with 1 × Protein Blocking Solution (Immunon Thermo Shandon, Pittsburgh, PA). Sections were stained with rat anti-mouse CD34 (Abcam) for 1 hour followed by goat anti-rat IgG-Biotin-labeled secondary antibody. Sections were then incubated with horseradish peroxidase-conjugated Streptavidin (Chemicon Australia, Melbourne, Australia). Slides were then incubated with AEC, counterstained with hematoxylin (BDH Laboratory), and mounted. As a measurement of angiogenesis, the microvessel density (i.e., the number of positive vessels in a given area) was determined. Quantification of microvessel density involved counting the number of CD34-positive vessels in four to six random 390-μm² fields at ×200 magnification for each tumor (n = 3) per treatment group.

Statistical analysis. All data was analyzed for significance by Student’s t test, except for the in vivo survival data, which was analyzed by log-rank Mantel-Cox test. Data from the microvessel density detection was analyzed for difference between treatment groups by a one-way ANOVA, where \( P < 0.05 \) was considered statistically significant.

Results

**Binding of monoclonal antibodies 806 and 528 and EGFR1 to cell lines.** We examined whether U87MG.Δ2-7 (which expresses the de2-7 EGFR) and U87MG.DK (which express a dead kinase form of the de2-7 EGFR) cells express similar levels of the various EGFR proteins. The EGFR1 antibody only recognizes the extracellular domain of the wt EGFR and does not bind to the de2-7 EGFR or the kinase dead form of de2-7 EGFR (7). EGFR1 bound both U87MG.Δ2-7 and U87MG.DK cells with similar intensity, confirming that these cell lines express comparable amounts of endogenous wt EGFR (Fig. 1). The mAb 528, which recognizes all of the EGFR subtypes, also stained both the U87MG.Δ2-7 and U87MG.DK cell lines to a comparable degree, showing that they express similar levels of total EGFR (Fig. 1). Finally, mAb 806, which in these cells only recognizes the de2-7 EGFR or its dead kinase equivalent (29), bound to each cell line with similar intensity indicating that both express comparable levels of either the de2-7 EGFR or dead kinase receptor (Fig. 1). Thus, the level of EGFR expression is similar in U87MG.Δ2-7 and U87MG.DK cell lines and therefore should not influence subsequent therapy studies.

We directly compared the relative binding of mAbs 806 and 528 in the two cell lines used in this study (i.e., U87MG.Δ2-7 and A431). Using a competitive ELISA, we have previously shown that mAbs 806 and 528 do not compete and can bind the EGFR simultaneously (30). However, we have previously shown by fluorescence-activated cell sorting (22) and immunoprecipitation (31) that whereas mAb 806 binds a high proportion of de2-7 EGFR (~50%), it only binds a small proportion of the wt EGFR only in cells overexpressing the receptor. Consistent with these previous observations, mAb 806 bound A431 cells, which overexpress the wt EGFR, at a lower level than mAb 528 (Fig. 1B). Likewise, mAb 528 bound more receptors on U87MG.Δ2-7 cells compared with mAb 806 (Fig. 1C). Again, this was expected as mAb 528 not only binds the de2-7 EGFR expressed in these cells but also the coexpressed wt EGFR, whereas mAb 806 can only bind the de2-7 EGFR in these cells.

**Treatment of established glioma xenografts with monoclonal antibodies 806 and 528.** We examined whether two mAbs with differing specificity for the EGFR displayed increased antitumor activity against glioma tumor xenografts when used together. Treatment of established U87MG.Δ2-7 glioma xenografts with

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**Fig. 1.** Flow cytometric analysis of U87MG.Δ2-7 and U87MG.DK cell lines. A, cells were stained with either an irrelevant IgG2b antibody or the EGFR1 antibody, mAb 806 or 528 as indicated. B, A431 cells were stained with an irrelevant isotype control (dark gray), mAb 528 (light gray), and mAb 806 (white) and analyzed by fluorescence-activated cell sorting. The dotted line is cells with secondary antibody alone. C, as for (B) except U87MG.Δ2-7 cells were used.
mAbs 806 and 528 in combination displayed significantly higher antitumor activity than either antibody alone (Fig. 2A). Whereas treatment with mAb 806 or 528 as single agents at day 20 after inoculation resulted in a reduction in overall tumor growth rate compared with treatment with vehicle (P < 0.005 and P < 0.003, respectively), combined treatment with both antibodies resulted in at least additive tumor inhibition that was significantly higher than mAb 528 (P < 0.001) and mAb 806 (P < 0.001) alone. The average tumor volume on the final day of therapy (day 20 after inoculation) was 1,850, 880, 800, and 30 mm³ for the vehicle, mAb 806, mAb 528, and combination treatment groups, respectively. Furthermore, 30% of U87MG.D2-7 xenografts underwent complete regression in the combination therapy group and had not recurred at day 30 after inoculation. No complete regressions were seen in any of the other treatment groups (Fig. 2A).

In the above experiment, the total amount of antibody given in the combination group was twice as much as the groups receiving only one mAb. Whereas it is typical to use this type of dose schedule in combination studies, we wanted to ensure that our observations were not simply due to the higher antibody dose. Combination therapy using 0.5 mg per injection of each antibody (1 mg of total antibody) displayed significantly higher antitumor activity than 1 mg per injection of mAb 806 (P < 0.01) or mAb 528 (P < 0.001) used as single agents when assessed on day 23 after inoculation (Fig. 2B). Thus, the enhanced activity of combination antibody therapy is not simply due to higher levels of antibody at the xenograft site. Previous reports have shown that the efficacy of EGFR-specific antibodies is reduced when used to treat large xenografts (32). Therefore, the efficacy of mAbs 806 and 528 combination therapy was examined against large established U87MG.D2-7 xenografts with a mean volume of 550 mm³ (Fig. 2C). Only xenografts treated with mAbs 806 and 528 in combination displayed a significant reduction in xenograft growth when compared with tumors treated with vehicle (P < 0.01; Fig. 2C). No significant difference in tumor growth rate was observed following treatment with mAb 806 (P = 0.40) or mAb 528 (P = 0.30). Indeed, the average tumor volume on day 26 after inoculation was 2,140, 2,080, 1,990, and 1,210 mm³ for the vehicle, mAb 806, mAb 528, and combination treatment groups, respectively (Fig. 2C). These results suggest that the combination of mAbs 806 and 528 results in greater than additive antitumor activity.
Mice bearing established U87MG.DK xenografts were also treated with mAbs 806 and 528 as single agents or in combination to ascertain whether the antibodies mediated the antitumor activity through immune effector function or signaling effects. On day 32 after inoculation, the growth rate of tumors treated with mAbs 806 and 528 as single agents or in combination did not differ from that of vehicle-treated xenografts. The average tumor volume was 1,170, 1,110, 1,260, and 1,220 mm$^3$ for vehicle, mAb 806, mAb 528, and combination therapy groups, respectively (Fig. 2D). Because both antibodies bind U87MG.D2-7 and U87MG.DK cells equivalently (Fig. 1), this result indicates that both mAbs 806 and 528 initiate their antitumor activity independent of immune effector function in this model.

**Survival analysis for mice treated with antibodies.** We also analyzed the data from the combination antibody therapy studies (Figs. 2A and C) with respect to survival. When used as single agents (three 0.5 mg injections per week for 2 weeks) to treat small established U87MG.D2-7 xenografts (i.e., mice shown in Fig. 2A), both mAbs 806 and 528 had a small benefit on survival ($P < 0.004$ and 0.01, respectively) with median survival being days 23 and 22, respectively, compared with day 20 for the control group (Fig. 2E). All mice receiving a combination antibody treatment survived to day 31 ($P < 0.0001$ versus control; $P < 0.002$ versus mAb 528 alone, and $P < 0.001$ versus mAb 806 alone; Fig. 2E), at which point the experiment was terminated for ethical reasons. Thus, combination therapy significantly improved survival in all mice with small established tumors compared with either antibody alone.

We also analyzed survival in the cohort of mice that were treated with antibody once their U87MG.D2-7 xenografts had reached an average of 500 mm$^3$ (i.e., mice shown in Fig. 2C). Consistent with the growth curves (Fig. 2C), neither mAb 806 nor mAb 528 used alone had any effect on survival (Fig. 2F) with median survival being day 22 for all three groups. In contrast at day 26, when the experiment with large xenografts had to be terminated for ethical reasons, 90% of mice had survived in the combination group. Therefore, combination antibody treatment greatly enhanced survival even in mice with large established xenografts providing further evidence of the synergistic nature of these two antibodies.

**Mechanism of synergistic antitumor activity.** To determine a mechanism for the synergistic antitumor activity of mAbs 806 and 528 in combination, we examined whether either antibody alone or in combination down-regulated the expression of de2-7 EGFR on the cell surface. U87MG.D2-7 cells were incubated at 37°C with antibodies for 30 minutes, lysed, and analyzed for de2-7 EGFR levels by immunoblotting. Whereas mAbs 806 and 528 alone had no effect on de2-7 EGFR expression, when used in combination, there was a dramatic decrease in the total de2-7 EGFR (Fig. 3A, top), suggesting that in combination the antibodies induce rapid down-regulation of the receptor. Whereas no decrease in the level of phosphorylation at Tyr$^{1173}$, the major activation site for the EGFR, was detected following treatment with mAb 806, a noticeable decrease was observed following mAb 528 treatment (Fig. 3A, second). However, the level of phosphorylation at Tyr$^{1173}$ was greatly reduced in the presence of both antibodies (Fig. 3A, second), compared with treatment with either antibody alone, which correlates with the decrease in total de2-7 EGFR. Consistent with the lack of synergistic antitumor activity seen when mAbs 806 and 528 were used together against U87MG.DK xenografts, this combination of antibodies did not induce down-regulation of the dead kinase de2-7 EGFR (Fig. 3A, third). As expected, there was virtually no phosphorylation of Tyr$^{1173}$ with the dead kinase receptor (Fig. 3A, bottom). Thus, the synergistic activity of mAbs 806 and 528 in combination seems related to their ability to cause extensive down-regulation of the de2-7 EGFR.

To determine whether these in vitro observations are mimicked in vivo, we examined the level of de2-7 EGFR in established U87MG.D2-7 and U87MG.DK xenografts 1 day after the third administration of mAbs 806 and 528. These experiments were done as described for Fig. 2B, where the total amount of antibody given was identical in all groups. The down-regulation of total de2-7 EGFR and decreased phosphorylation of Tyr$^{1173}$ following treatment with mAbs 806 and 528 in combination was observed in antibody-treated U87MG.D2-7 xenografts (Fig. 3B, top and second). In contrast to the in vitro assay, mAb 806 alone did block the phosphorylation of Tyr$^{1173}$ in treated xenografts, suggesting that its effects on signaling inhibition maybe more pronounced in vivo.

p27$^{kip1}$ is a negative regulator of G1-S phase transition that functions by inhibiting cyclin-Cdk kinase activity (33). Treatment of U87MG.D2-7 xenografts with mAb 806 or 528 alone produced a measurable increase in p27$^{kip1}$ expression compared with tumors treated with PBS (Fig. 3B, third). Treatment with both antibodies in combination caused an additive increase in the level of p27$^{kip1}$, consistent with the down-regulation of total de2-7 EGFR. As expected, U87MG.DK xenografts (Fig. 3C) treated with the combination of antibodies did not show down-regulation of de2-7 EGFR (Fig. 3C, top) or changes in the level of p27$^{kip1}$ (Fig. 3C, third) compared with xenografts treated with PBS or either antibody alone.

To determine if antibody mediated down-regulation of the de2-7 EGFR combined with the up-regulation of p27$^{kip1}$ has an antiproliferative effect on xenografts, we analyzed the percentage of Ki67-positive cells from treated mice. These experiments were also done as described for Fig. 2B, where the total amount of antibody given was identical in all groups. U87MG.D2-7 xenografts treated with vehicle had a PI of 34.6% (Fig. 4A). In contrast, U87MG.D2-7 xenografts treated with either mAb 806 or 528 had lower PI of 20.1% and 19.6%, respectively ($P < 0.001$). U87MG.D2-7 xenografts treated with both mAbs 806 and 528 in combination had a PI of 11.3%, which was significantly lower than the vehicle, mAb 806 or 528 treatments ($P < 0.001$ for all groups; Fig. 4A). Consistent with its slower growth rate, the PI of U87MG.DK xenografts treated with vehicle was 18.7% (Fig. 4A). Treatment with mAb 806 (PI = 18.8%) or 528 (PI = 18.3%) had no effect on the proliferation of U87MG.DK xenografts. Significantly, the combination treatment did not have any effect (PI = 19.1%) on the U87MG.DK xenografts (Fig. 4A). Interestingly, analysis of the apoptotic index through terminal deoxynucleotidyl transferase–mediated nick-end labeling staining (28) showed an extremely low level of apoptosis that did not differ between treatment and control groups at this early time point (data not shown).

To further assess the in vivo mechanisms underlying the antitumor activity of mAbs 806 and 528 when used in combination, the extent of intratumoral microvessel density of U87MG.D2-7 xenografts treated as in Fig. 2B, was assessed by immunostaining xenograft sections for CD34 (Fig. 4B). The
number of CD34-positive microvessels in xenografts treated with PBS was 34.2 ± 10.8. Following treatment with mAbs 806 or 528 as single agents, the number of mean CD34-positive microvessels was 35.2 ± 4.8 and 37.6 ± 8.5, respectively (Fig. 4B). These values were not statistically different to that observed for PBS-treated tumors. However, following combined mAbs 806 and 528 therapy, there was a statistically significant reduction of 46% in the number of CD34-positive vessels compared with PBS-treated tumors (18.4 ± 5.8; P < 0.0001; Fig. 4B), indicating that the inhibition of U87MG.D2-7 tumors in vivo following combination antibody therapy is partly the result of the blockade of angiogenic progression. Both the subclass control sections and the negative control sections did not stain positive for CD34.

Treatment of established A431 xenografts with monoclonal antibodies 806 and 528. Given the efficacy of antibody combination therapy against U87MG.D2-7 xenografts, we elected to examine its efficacy against A431 tumors. A431 cells contain an amplification of the wt EGFR gene and overexpress the receptor on the cell surface. Combination therapy with mAbs 806 and 528 displayed at least additive antitumor activity against established A431 xenografts. At day 20 after tumor inoculation, the A431 xenografts treated with both antibodies had a mean tumor volume of 15 mm³ which was significantly lower than vehicle (1,016 mm³, P < 0.001), mAb 806 alone (473 mm³, P < 0.001), and mAb 528 alone (372 mm³, P < 0.001) treatment groups (Fig. 5A and B). Furthermore, combined treatment with mAbs 806 and 528 caused complete regression in 60% of the tumors, which again is indicative of a synergistic antitumor effect (Fig. 5B). Histologic examination at day 28 after inoculation revealed the complete absence of tumor cells in the majority of regressed xenografts (data not shown). No complete regressions were seen in any of the other treatment groups.

We also treated large A431 xenografts with the combination of mAbs 806 and 528 (Fig. 5C). A431 xenografts were allowed to grow to ~515 mm³ at which time antibody therapy commenced. Treatment with mAb 806 alone did not inhibit A431 tumor growth rate when compared with vehicle-treated controls at day 26 after inoculation (P = 0.19), whereas treatment with mAb 528 alone did have an antitumor effect (P < 0.001). However, treatment with both antibodies in combination resulted in a greater than additive reduction in A431 xenograft growth rate compared with vehicle (P < 0.001), mAb 806 alone (P < 0.001), and mAb 528 alone (P < 0.01) treatment groups. At day 26, when vehicle-treated animals were sacrificed, the mean tumor volume was 1,810, 2,150, 850, and 360 mm³ in the vehicle, mAb 806, mAb 528, and combination treatment groups, respectively (Fig. 5C).

Systemic treatment of intracranial glioma xenografts with monoclonal antibodies 806 and 528. To further test the efficacy...
of mAbs 806 and 528 in combination, we treated mice bearing i.c. U87MG.D2-7 glioma xenografts with i.p. injection of antibody (Fig. 6A). Animals treated with vehicle had a median survival of 14.8 days, whereas systemic treatment with either 0.5 mg mAb 806 or 528 increased the median survival to 19.2 (P < 0.003) and 17.6 (P < 0.009) days, respectively (Fig. 6A). Treatment of animals with 0.5 mg of mAbs 806 and 528 in combination significantly prolonged survival to a median of 25.7 days (P < 0.001 compared with all groups).

Animals bearing i.c. U87MG.DK xenografts treated with vehicle had a median survival of 24.2 days, whereas those treated with mAbs 806 and 528 in combination significantly prolonged survival to a median of 25.7 days (P < 0.001 compared with all groups). Animals bearing i.c. U87MG.DK xenografts treated with vehicle had a median survival of 14.8 days, whereas systemic treatment with either 0.5 mg mAb 806 or 528 increased the median survival to 19.2 (P < 0.003) and 17.6 (P < 0.009) days, respectively (Fig. 6A). Treatment of animals with 0.5 mg of mAbs 806 and 528 in combination significantly prolonged survival to a median of 25.7 days (P < 0.001 compared with all groups).

Whole brain sections from mice with i.c. U87MG.D2-7 xenografts were histologically analyzed at the completion of antibody therapy (i.e. day 14 after inoculation). Consistent with the survival data, combined treatment with mAbs 806 and 528 dramatically reduced the volume of i.c. U87MG.D2-7 tumors in comparison to those treated with vehicle, mAb 806 alone, or mAb 528 alone (Fig. 6C). Combination antibody treatment of U87MG.DK i.c. tumors did not produce a reduction in tumor volume compared with treatment with vehicle (Fig. 6D).

**Discussion**

Previous reports have shown the *in vitro* and *in vivo* antitumor activity of mAb 528 against a variety of malignant epithelial cell lines overexpressing the wt EGFR (27, 34, 35). Because mAb 528 and similar antibodies such as IMC-C225 are potent EGFR ligand antagonists, it has been presumed that their antitumor activity is predominantly mediated by blocking the ligand activation of the EGFR (36, 37). Our data showing that mAb 528 has significant antitumor activity against xenografts expressing the ligand-independent but constitutively active de2-7 EGFR shows that this antibody mediates its inhibitory effects through multiple mechanisms and not simply ligand blockade. The antitumor activity of mAb 528 against U87MG.D2-7 xenografts was not directly mediated through its effect on the endogenous wt EGFR coexpressed in these cells, as mAb 528 did not inhibit the growth of parental U87MG, or U87MG.DK xenografts both of which express similar levels of the wt EGFR. Furthermore, the lack of mAb 528 activity against U87MG.DK xenografts suggests that antibody effector function does not have a significant role in initiating the antitumor activity. To the best of our knowledge, this is the first report demonstrating that an antibody to the wt EGFR also has direct antitumor activity against de2-7 EGFR—expressing xenografts and partially addresses the concern that expression of de2-7 EGFR may cause resistance to EGFR antibody therapy.

Treatment of established U87MG.D2-7 xenografts with a combination of mAbs 806 and 528 resulted in additive, and in some cases synergistic, tumor inhibition leading to complete regression of some of these aggressive tumors. Furthermore, the almost complete resistance of large U87MG.D2-7 xenografts to either antibody alone was overcome by using the antibodies in combination. Significantly, this increased efficacy seen with...
Combination Therapy with Multiple EGFR Therapeutics

combination antibody therapy was also observed in our i.c. model. Whereas the increase in survival was relatively modest (i.e., 11 days), it should be noted that i.c. U87MG.Δ2-7 tumors grow extremely rapidly with control animals dying just 15 days after inoculation with 100,000 cells. Indeed, combination therapy should be more effective against less aggressive tumors, as was the case when we evaluated mAb 806 as a single agent (29). Importantly, the antitumor effect of both antibodies was seen following systemic administration, extending our previous data with mAb 806 alone (29). This observation suggests it might be possible to use systemic antibody therapy in glioma patients with advanced disease as there is usually localized disruption of the blood/brain barrier in these patients.

The in vivo consequences of EGFR blockade by mAbs such as 528 has not been fully elucidated, although recent reports suggest that these antibodies switch off key EGFR signaling pathways leading to decreased proliferation, increased apoptosis, and the inhibition of proangiogenic growth factors, all of which can contribute to the antitumor effects seen (38). Analysis of mAb 806- and mAb 528-treated U87MG.Δ2-7 xenografts showed that these antibodies caused a significant reduction in tumor cell proliferation compared with PBS-treated tumors which correlates with the in vivo growth rate of tumors treated with both antibodies in combination. This is most probably due to the significant down-regulation and degradation of de2-7 EGFR, which was observed following combination antibody therapy both in vitro and in vivo. This observation is in line with a recent report showing similar effects following treatment of ErbB2-overexpressing breast cancer cells with a combination of the anti-ErbB2 antibodies Herceptin and 2C4 (39). Combination Herceptin-2C4 treatment reduced ErbB2 levels within 24 hours to a greater degree than either agent alone (39). As both the ErbB2 and de2-7 EGFR do not bind ligand and are internalized respectively at a slower rate (40–42) or not at all (43) compared with wt EGFR, down-regulation of these receptors may contribute to signal attenuation and subsequent growth inhibition. Indeed, our data shows that combination therapy with both antibodies leads to down-regulation and degradation of de2-7 EGFR, which would lead to attenuation of the constitutive de2-7 EGFR signal.

A reduction in the level of receptor signaling would in turn have consequences on the proliferative capability of cells. Indeed, previous reports, investigating the ability of anti-EGFR agents to block EGFR signaling through blockade of ligand binding or kinase activation, have shown that these agents reversibly arrest cells in G1 phase of the cell cycle by modifying the expression of key proliferative/antiproliferative signaling molecules such as p27KIP1, leading to blockade of proliferation (44–48). In particular, the expression of p27KIP1 is thought to be required for growth inhibition when the EGFR pathway is blocked in cell lines dependent on the EGFR for their viability (48). Similarly, the enhanced tumorigenicity of U87MG.Δ2-7 cells was directly related to de2-7 EGFR-mediated down-regulation of p27KIP1, through activation of the phosphatidylinositol 3-kinase/Akt pathway (49), identifying a potential pathway for therapeutic intervention. Indeed, U87MG.Δ2-7 xenografts treated with either mAb 528 or 806 showed specific increase in p27KIP1 expression compared with PBS-treated xenografts. Combination antibody therapy also led to enhanced up-regulation of p27KIP1, which was greater than that observed following single-agent therapy. Furthermore, as mAb 806 does not bind to the endogenous wt EGFR expressed in these cells, increased p27KIP1 expression following mAb 806 therapy would be directly attributable to the interaction between this antibody and the de2-7 EGFR. Therefore, the additive up-regulation of p27KIP1 following combination antibody therapy would not occur simply through dual targeting of the wt EGFR but rather from specific targeting of both subsets of receptor.

Taken as a whole, the down-regulation of de2-7 EGFR and concurrent up-regulation of p27KIP1 following combination

Fig. 5. Combination treatment of A431 xenografts with mAbs 806 and 528 in an established model. A, mice (n = 5) bearing A431 xenografts were treated with four i.p. injections of PBS (○), 0.5 mg of mAb 806 (●), 0.5 mg of mAb 528 (▲) as single agents, or 0.5 mg of each antibody in combination (□) on days 8, 10, 12, and 14, and 16 once tumors had reached 70 mm3. B, representative mice from the PBS and combination antibody treatment groups in (A) 1 day after the final therapy injection (day 15). C, treatment of mice bearing A431 xenografts as in (A) on days 13, 14, 15, and 16 once tumors had reached 515 mm3.
antibody therapy led to an additive antiproliferative effect and decreased intratumoral microvessel formation. Furthermore, the lack of down-regulation of the dead kinase variant of the de2-7 EGFR following combination antibody therapy, together with no changes in p27kip1 expression or proliferative index correlate with the lack of therapeutic efficacy in vivo and highlight the importance of de2-7 EGFR kinase activity in mediating the inhibitory effects of these antibodies.

Finally given the significant decrease in proliferation and inhibition of blood vessel formation in U87MG Δ2-7 xenografts treated with mAbs 806 and 528 in combination, together with the up-regulation of p27 that inhibits G1-to-S phase transition, it is interesting that we do not see significant differences in apoptosis between treatment groups. However, given that these observations represent mechanisms of tumor cell inhibition early in the therapy cycle (1 day after the third injection of antibody), it is likely that changes in apoptotic level may become evident following prolonged therapy. Indeed, our unpublished data show that continued antibody therapy is able to cause a significant increase in apoptosis compared with control-treated tumors.6 Similarly, although our data do not show a difference in microvessel density between xenografts treated with mAb 806 or 528 as single agents compared with PBS, it is possible that such observations would be more likely following prolonged antibody therapy. A previous report by Mishima et al. reported a 30% decrease in tumor vascularity in mAb 806–treated tumors compared with control tumors (29). However, this occurred after prolonged therapy, where antibody was given for 14 consecutive days again highlighting the possibility that such events may become more evident following sustained therapy over longer time frames.

Greater than additive antitumor activity was also observed following treatment of established A431 xenografts with a combination of mAbs 806 and 528. This observation is somewhat remarkable given that mAb 528 recognizes the majority of EGFR expressed on A431 cells and mAb 806 binds <10% of the receptors (22). Recently, we have shown that mAb 806 recognizes a transitional form of the EGFR and prevents the formation of signaling-capable EGFR dimers (23, 24). Thus, whereas mAb 806 only binds a low percentage of the EGFR at any given time, over an extended period of time, it would be capable of inhibiting a substantial proportion of EGFR signaling which in turn generates an antitumor effect in vivo. Thus, the antitumor mechanism mediated by mAb 806 is clearly distinct to the ligand-inhibiting activity of mAb 528. Therefore, these antibodies inhibit wt EGFR signaling by different modes of action, which in the case of A431 xenografts, results in greater than additive tumor inhibition.

The most important outcome of our experiments is that single EGFR therapeutics do not inhibit all the functions associated with the de2-7 or the overexpressed wt EGFR; thus, it may be necessary to target the EGFR with multiple therapeutics. Indeed, our data suggests that the development of novel therapeutics to the EGFR should continue to identify new agents with unique properties. Given that we and others recently determined the crystal structure of the extracellular domain of the EGFR, it should be possible to identify regions of the receptor that represent potential targets (50, 51). Finally, if there are some aspects of EGFR function that are not inhibited by antibodies such as mAb 528, it may explain why some EGFR therapeutics have had less clinical success than anticipated (52). Therefore, combination therapy with mAb 806 and agents such as IMC-C225 or Iressa may increase the response rate seen in patients. Consequently, we are currently developing a chimeric version of mAb 806 for use in clinical trials.

6 Unpublished data.
References


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Treatment of Human Tumor Xenografts with Monoclonal Antibody 806 in Combination with a Prototypical Epidermal Growth Factor Receptor–Specific Antibody Generates Enhanced Antitumor Activity

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