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(71) Applicant(s)
Ludwig Institute for Cancer Research

(72) Inventor(s)
Johns, Terrance Grant;Furnari, Frank;Cavenee, Webster;Scott, Andrew

(74) Agent / Attorney
Spruson & Ferguson, L 35 St Martins Tower 31 Market St, Sydney, NSW, 2000

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- (71) Applicant (for all designated States except US): LUDWING INSTITUTE FOR CANCER RESEARCH [US/US]; 605 Third Avenue, New York, NY 10158 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): CAVENEE, Webster [US/US]; 9500 Gilman Drive, Cellular And Molecular Medicine-East, 3041, La Jolla, CA 92093-0660 (US). FURNARI, Frank [US/US]; 9500 Gilman Drive,

Cellular And Molecular Medicine-East, 3041, La Jolla, CA 92093-0660 (US). **JOHNS, Terrance, Grant** [AU/AU]; Level 6, Harold Stokes Building, 145-163 Studley Road, Heidelberg, VIC 3084 (AU). **SCOTT, Andrew** [AU/AU]; Level6, Harold Stokes Building, 145-163 Studley Road, Heidelberg, VIC-3084 (AU).

(74) Agent: **DIETZEL, Christine, E.**; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).

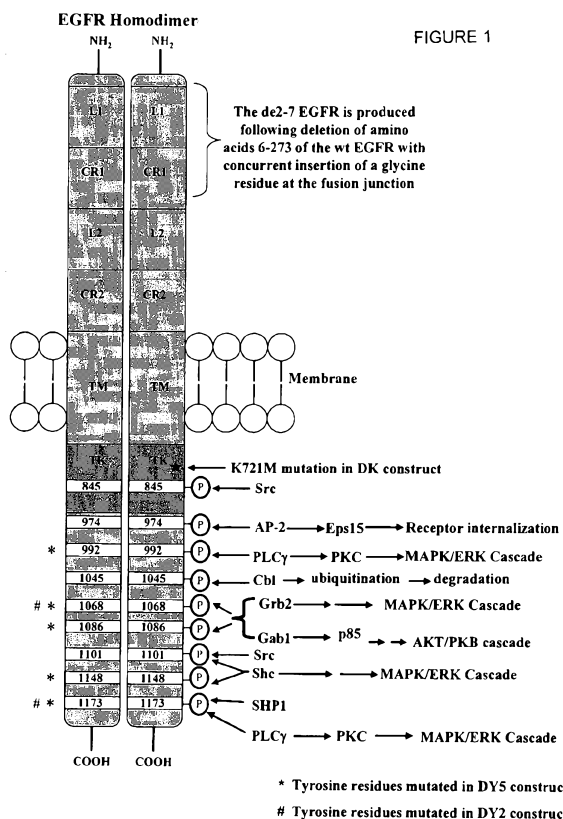
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(54) Title: TREATMENT METHOD USING EGFR ANTIBODIES AND SRC INHIBITORS AND RELATED FORMULATIONS

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(57) Abstract: The present invention relates to the treatment of EGFR-mediated disease, particularly cancer by inhibiting or blocking EGFR and src in combination or simultaneously. The invention relates to treatment, prevention, or modulation of cancer, particularly EGFR-mediated disease, with one or more EGFR modulator and src inhibitor in combination. The invention further relates to the treatment of cancer with anti-EGFR antibodies and src inhibitors. Methods and compositions for treatment of cancer with the antibody anti-EGFR mAb806 in combination or series with a src inhibitor or src inhibitors are described.



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TREATMENT METHOD USING EGFR ANTIBODIES AND SRC INHIBITORS AND RELATED FORMULATIONS

FIELD OF THE INVENTION

[0001] The present invention relates to the treatment of EGFR-mediated disease, particularly cancer. Methods for treatment of cancer using combinations of EGFR modulators, particularly EGFR antibody(ies), and src inhibitors are provided. Methods and combinations of MAb806 antibody and src inhibitors are provided.

BACKGROUND OF THE INVENTION

[0002] Targeted cancer therapy is designed to disrupt the function of specific molecules needed for carcinogenesis and tumor growth and thus either kills or prevents the growth of cancer cells (Ji H et al (2006) Cell Cycle 5(18):2072-2076 Epub 2006 Sep15). In contrast to conventional cytotoxic chemotherapy, such targeted cancer therapies may be more effective and less harmful to normal cells. A main effort in the targeted cancer therapy field has been the development of agents that target the epidermal growth factor receptor (EGFR). EGFR is a member of the ErbB family of closely related receptors including EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4). Activation of EGFR leads to receptor tyrosine kinase activation and a series of downstream signaling events that mediate cellular proliferation, motility, adhesion, invasion, and resistance to chemotherapy as well as inhibition of apoptosis, processes that are crucial to the continual proliferation and survival of cancer cells.

[0003] As expression of the EGFR VIII mutant receptor is restricted to tumor cells, it represents a highly specific target for antibody therapy. Accordingly, both polyclonal and monoclonal antibodies specific to the unique peptide of de2-7 EGFR have been generated. A series of mouse mAbs, isolated following immunization with the unique

de2-7 peptide, all showed selectivity and specificity for the truncated receptor and targeted de2-7 EGFR positive xenografts grown in nude mice (Wikstrand CJ et al (1995) Cancer Res 55:3140-3148; Okamoto, S et al (1996) Br J Cancer 73:1366-1372; Hills D et al (1995) Int J Cancer 63:537-543; Reist CJ et al (1997) Cancer Res 57:1510-1515; Reist CJ et al (1995) Cancer Res 55:4375-4382; U.S. Patent 5,401,828). Examples of anti-EGFR vIII antibodies include ABX-EGF (panitumumab), DH8.3, L8A.4, and Y10.

[0004] MAb806 is a novel murine antibody, originally raised to recognize the unique truncation mutant, EGFRvIII using whole cells expressing EGFR vIII mutant as immunogen. Importantly, the epitope recognized by mAb806 is not accessible in inactive wild-type (*wt*) EGFR, but is exposed in a transitional form of wt EGFR in cells with overexpression of EGFR, and expression of EGFRvIII. MAb806 binds to an epitope present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but recognizes an epitope distinct from the mutant's junctional peptide LEEKKGNVYVTDH. The epitope studies are supported by immunohistochemical studies demonstrating that the 806 antibody binds to epitopes present in gliomas, as well as a broad range of epithelial cancers, but not to normal human tissues. These and other preclinical data suggest that mAb806 might have a different spectrum of clinical activity and side effect profile distinct from cetuximab and other anti-EGFR antibodies. In xenograft models, mAb806 has exhibited a potent anti-tumor activity with no targeting of normal tissues. Thus, the unique targeting capabilities of mAb806 represent a new paradigm for cancer-specific molecularly targeted therapy.

[0005] The non-receptor protein tyrosine, Src, is a 60-kDa protein that is a member of a nine-gene family, including Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk, and Yrk, that plays a critical role in the regulation of many cellular processes, such as proliferation, differentiation, migration, adhesion, invasion, angiogenesis, and immune function (Yeatman TJ. (2004) Nat Rev Cancer 4(6):470-80; Frame MC. (2004) J Cell Sci 117:989-98). The Src family kinase contains a poorly conserved domain and three conserved Src homology domains: SH2, SH3, and SH1 or protein tyrosine kinase domain. Critical to the regulation of Src is a COOH-terminal tyrosine (Y530) that, when

phosphorylated by C-terminal Src kinase (Csk), leads to a more inactive Src conformation. Src interacts with many proteins, depending on the input signal. It further assumes its active conformation through dephosphorylation of Y530 and autophosphorylation of Y418. Src also associates with structural and signaling proteins, and the resulting complexes are critical to Src's role in diverse cellular processes. Src has been reported to be overexpressed or aberrantly activated in a number of cancers, such as colon, breast, melanomas, ovarian cancer, gastric cancer, head and neck cancers, pancreatic cancer, lung cancer, brain cancers, and blood cancers (Dehm SM and Bonham K (2004) *Biochem Cell Biol* 2004;82:263–74). There are several known small molecule inhibitors of src and some have entered clinical trials, for example dasatinib (BMS354825), AZD-0530, SKI-606, PP1 (4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine), PP2 (4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine), PD166326.

[0006] There is a clinical need for enhanced, more efficacious and more broadly effective treatment protocols for EGFR-mediated disease including cancer.

[0007] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0008] The invention relates to the discovery that alteration of src expression or activity enhances the efficacy of anti-EGFR therapies. In particular alteration of src expression or activity dramatically enhances anti-EGFR antibody efficacy, particularly the activity of mAb806 antibody.

[0009] The invention relates to the combination of EGFR and src inhibitors for treatment of cancer or other EGFR-mediated disease.

[0010] The invention further provides a method of treating EGFR-mediated cancer in a mammal comprising administering to said mammal a src inhibitor and anti-EGFR

antibody, either in combination, simultaneously, or in series, one after the other. In one aspect, the src inhibitor is a tyrosine kinase inhibitor. In one aspect, the anti-EGFR antibody is MAb806.

[0011] In a particular embodiment of the method the anti-EGFR antibody is mAb806 antibody or an active fragment thereof. MAb806 includes murine antibody, recombinant antibody or a humanized antibody.

[0012] The EGFR-mediated cancer may be selected from glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder cancer. The cancer may further be selected from colon, breast, melanomas, ovarian cancer, gastric cancer, pancreatic cancer, brain cancers, and blood cancers. In particular, the cancer may be glioma.

[0013] The invention provides a method of treating cancer in a mammal comprising administering to said mammal a src inhibitor and anti-EGFR antibody, wherein said src inhibitor and anti-EGFR antibody are administered simultaneously, in combination, or one after another series. In an aspect of the method, the anti-EGFR antibody is an antibody which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and not detectable in normal cells. In a particular such aspect, the anti-EGFR antibody is mAb806 or an active fragment thereof.

[0014] In the method(s), the src inhibitor may be selected from dasatinib (BMS354825), AZD-0530, SKI-606, PP1 (4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine), PP2 (4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine), and PD166326. In the method(s), the src inhibitor is particularly a tyrosine kinase inhibitor. In a particular embodiment of the method, the src inhibitor is dasatinib and the anti-EGFR antibody is mAb806.

[0015] The cancer may be selected from glioblastoma, head and neck cancer, pancreatic

cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, bladder cancer, colon cancer, melanomas, gastric cancer, pancreatic cancer, brain cancers, and blood cancers.

[0016] The invention provides a method for blocking or reducing tumor growth of an EGFR-mediated cancer in a mammal comprising administering to said mammal a src inhibitor and anti-EGFR antibody, wherein said src inhibitor and anti-EGFR antibody are administered simultaneously, in combination, or one after another in series. In a particular such method, the anti-EGFR antibody is an antibody which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and not detectable in normal cells. The anti-EGFR antibody is particularly mAb806 or an active fragment(s) thereof.

[0017] The invention provides a method for blocking or reducing tumor growth of an EGFR-mediated cancer in a mammal comprising administering to said mammal a src inhibitor and anti-EGFR antibody, wherein the src inhibitor is dasatinib and the anti-EGFR antibody is mAb806.

[0018] The EGFR-mediated cancer may be selected from glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder cancer.

[0019] The invention further provides a method of enhancing the effectiveness or activity of an anti-EGFR antibody in a mammal comprising administering to said mammal a combination of the anti-EGFR antibody and a src inhibitor. The src inhibitor may be selected from dasatinib (BMS354825), AZD-0530, SKI-606, PP1 (4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine), PP2 (4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine), and PD166326. The anti-EGFR antibody is particularly an antibody which recognizes an EGFR epitope which is found in

tumorigenic, hyperproliferative or abnormal cells and not detectable in normal cells. In a particular such aspect, the anti-EGFR antibody is mAb806.

[0020] The invention further relates to pharmaceutical composition(s) comprising an anti-EGFR antibody and one or more src inhibitor in a pharmaceutically acceptable carrier or diluent. The compositions included are compositions wherein the anti-EGFR antibody is an antibody which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and not detectable in normal cells. In a particular such composition, the anti-EGFR antibody is mAb806.

[0020a] In an embodiment the invention relates to a method of treating cancer in a mammal comprising administering to said mammal the src inhibitor dasatinib (BMS354825) and an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNVVTDH, wherein said src inhibitor dasatinib (BMS354825) and said anti-EGFR antibody or antigen-binding fragment thereof are administered simultaneously, in combination, or one after another in series.

[0020b] In an embodiment the invention relates to a method for blocking or reducing tumor growth of an EGFR-mediated cancer in a mammal comprising administering to said mammal the src inhibitor dasatinib (BMS354825) and anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and not is detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNVVTDH, wherein said src inhibitor dasatinib (BMS354825) and anti-EGFR antibody are administered simultaneously, in combination, or one after another in series.

[0020c] In an embodiment the invention relates to a method of enhancing the effectiveness or activity of an anti-EGFR antibody or an antigen-binding fragment thereof in a mammal comprising administering to said mammal a combination of the anti-EGFR antibody or antigen-binding fragment thereof, which recognizes an EGFR epitope which is

found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, and the src inhibitor dasatinib (BMS354825).

[0020d] In an embodiment the invention relates to a pharmaceutical composition comprising an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, and the src inhibitor dasatinib in a pharmaceutically acceptable carrier or diluent.

[0020e] In an embodiment the invention relates to use of the src inhibitor dasatinib (BMS354825) or an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, for the manufacture of a medicament or medicaments for the treatment of cancer in a mammal, wherein in use of said medicament(s) said src inhibitor dasatinib (BMS3454825) and said anti-EGFR antibody or antigen-binding fragment thereof are administered simultaneously, in combination, or one after another in series.

[0020f] In an embodiment the invention relates to use of the src inhibitor dasatinib (BMS354825) or an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, for the manufacture of a medicament or medicaments for blocking or reducing tumor growth of an EGFR-mediated cancer in a mammal, wherein in use of said medicament(s) said src inhibitor dasatinib (BMS3454825) and said anti-EGFR antibody or antigen-binding fragment thereof are administered simultaneously, in combination, or one after another in series.

[0021] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIGURE 1. **Schematic representation of the EGFR.** The extracellular region deleted in the de2-7 EGFR is identified by parenthesis. The dead kinase version of the de2-7 EGFR contains a single point mutation (K→M) at position 721. The DY2 version of the de2-7 EGFR has Y→F mutations at residues 1068 and 1173, while the DY5 variant also has these substitutions plus 992, 1086 and 1148.

[0023] FIGURE 2. **Sensitivity of different xenografts to EGFR-specific antibodies.** Xenografts were established by injection of 3×10^6 cells in both flanks of nude BALB/c mice. Antibody therapy commenced when xenografts reached an approximate mean volume of 100 mm^3 . Mice were treated with 1 mg of mAb 528 (left panel) or mAb 806 (right panel) three times per week for two weeks (i.e. a total of 6 injections). Data are expressed as mean tumor volume \pm SE.

[0024] FIGURE 3. **Xenograft growth curves for U87MG based cell lines.** Xenografts were established by injection of 1×10^6 cells in both flanks of nude BALB/c mice in order to determine growth curves. Data are expressed as mean tumor volume \pm SE.

[0025] FIGURE 4A and 4B. ***In Vitro* Phosphorylation of de2-7 EGFR Variants in U87MG.Δ2-7, U87MG.DK and U87MG.DY5 cells.** A, the de2-7 EGFR protein was immunoprecipitated with mAb 806, mAb 528 or an irrelevant isotype matched control antibody and resulting samples immunoblotted. All de2-7 EGFR variants were positive for phosphorylation at Y1045, the major site associated with ubiquitination and degradation (top panel). While the de2-7 EGFR was constitutively phosphorylated at position Y1173, both the DK and DY5 variants were negative for phosphorylation at this site as expected (middle panel). The presence of EGFR was confirmed using the rabbit c-terminal polyclonal antibody to the EGFR (lower panel). This c-terminal antibody did not recognize the DY5 variant because it contains a Y1068F mutation, which turns out to be a critical residue for antibody binding. Thus, the presence of total DY5 protein was confirmed in (B) by immunoblotting with mAb 806.

[0026] FIGURE 5A-5D. **U87MG cells expressing high levels of de2-7 EGFR.** U87MG.Δ2-7 cells were FACS sorted into low (L), medium (M) and high (H) expressing populations. A, Cells were lysed following 36 h of serum starvation and analyzed by immunoblotting for de2-7 expression (C13) and tyrosine phosphorylation (4G10) of the de2-7 EGFR. Levels of phosphorylation correlated with de2-7 EGFR. B, Parental U87MG, U87MG-L, U87MG-M and U87MG-H xenografts were established by injection of 1×10^6 cells in both flanks of nude BALB/c mice in order to determine growth curves. Data are expressed as mean tumor volume \pm SE. C, Tumors from (B) were analyzed by immunoblotting for expression of de2-7 EGFR (C13). D, Mice with U87MG-H xenografts were treated with 1 mg of mAb 528 or mAb 806 three times per week for two weeks (days 4, 6, 8, 11, 13 and 15). Data are expressed as mean tumor volume \pm SE.

[0027] FIGURE 6A-6C. **Treatment of NR6.Δ2-7 xenografts with EGFR-specific antibodies.** Xenografts were established by injection of 3×10^6 cells in both flanks of nude BALB/c mice. Antibody therapy commenced when xenografts reached an approximate mean volume of 100 mm^3 . Mice were treated with 1 mg of mAb 806 (A) or mAb 528 (B) three times per week for two weeks (days 22, 25, 29, 32, 36 and 39) or with

mAb 528 (C) two times per week for three weeks (days 27, 30, 34, 37, 41 and 44). Data are expressed as mean tumor volume \pm SE.

[0028] FIGURE 7A-7C. Interaction between de2-7 EGFR and Src. (A) Cells were serum starved overnight prior to treatment with 10 μ M PP1 or PP2 or vehicle (DMSO) for 30 minutes or 24 h prior to immunoprecipitation with mAb528, mAb806 or an irrelevant isotype control. Immunoblotting was performed with an antibody specific for Y845 of the EGFR, while total de2-7 EGFR was visualized with the rabbit c-terminal polyclonal antibody. Results shown are representative of four independent experiments. (B) U87MG. Δ 2-7_{vector control} and U87MG. Δ 2-7_{DNSrc} xenografts were established by injection of 1×10^6 cells in both flanks of nude BALB/c mice in order to determine growth curves. Data are expressed as mean tumor volume \pm SE. (C) U87MG. Δ 2-7_{DNSrc} xenografts were established by injection of 3×10^6 cells in both flanks of nude BALB/c mice. Antibody therapy commenced when xenografts reached an approximate mean volume of 100 mm³. Mice were treated with 1 mg mAb 806 three times per week for two weeks (days 18, 20, 22, 25, 27 and 29). Data are expressed as mean tumor volume \pm SE.

[0029] FIGURE 8A and 8B. Co-localization of internalized mAb 806-Cy3 and EEA1 or lgp-120 in U87MG. Δ 2-7 Cells. (A) Cells seeded on glass coverslips were pre-incubated with mAb 806-Cy3 (*red*) at 4°C (0 min). Internalization was stimulated by incubation at 37°C for 10, 20 and 30 mins. Cells were fixed and permeabilized, then stained with anti-EEA1 followed by Cy2-conjugated donkey anti-mouse antibody (*green*). Co-localization is indicated by *yellow* in the merged images (*arrows*). Scale bar = 20 μ m. (B) Cells were transiently transfected with lgp-120 tagged with GFP (lgp-120-GFP; *green*). Positively transfected cells are shown in the lgp-120-GFP panel and by green arrowheads. Following transfection, cells were incubated with mAb 806-cy3 at 4°C (*red*; 0 min), prior to induction of internalization by incubating at 37°C for 30, 60 and 120 min. Samples were subsequently fixed and co-localization of mAb 806-Cy3 and lgp-120-GFP are indicated by the presence of *yellow* in the merged images (*white arrows*). Scale bar = 10 μ m.

[0030] FIGURE 9A-9F. **Electron microscopic analysis of clathrin mediated endocytosis and intracellular trafficking of mAb 806 following binding to de2-7 EGFR in U87MG.Δ2-7 cells.** Gold particles (mAb 806-Au; *arrowheads*) were readily detected in clathrin coated pits (A-B) and vesicles (C) following induction of internalization for 5 mins. No gold particles were present in structures resembling caveolae (*open arrowheads*) (D). After 10-15 mins of internalization, gold particles were detected in tubular vesicular structures resembling early endosomes (E). After longer periods of internalization, gold particles were seen in multivesicular bodies (F). *Scale bar* = 100 nm.

[0031] FIGURE 10. **Internalization of mAb 806 and mAb 528 in NR6.Δ2-7 Cells.** Cells were pre-incubated with mAb 806-Cy3 (**left panel**) or mAb 528-Cy3 (**right panel**) at 4°C (0 min), prior to incubation at 37°C for varying periods of time to induce internalization. Images representing 15, 30 and 60 mins incubation at 37°C are shown. Staining with both antibodies prior to internalization was associated with membrane junctions between cells (*blue arrowhead*) and focal adhesions (*red arrowhead*), while some cells showed very little membrane staining (*yellow arrowhead*). Internalized antibody at later time points is indicated by white arrows. *Scale bar* = 20 μm.

[0032] FIGURE 11. **Schematic representation of the interaction of the de2-7 EGFR variants with other cellular components.** The de2-7 EGFR has an active kinase and therefore can autophosphorylate, transphosphorylate or be the target of phosphorylation by other kinases. In contrast, the dead kinase de2-7 EGFR can only be the target of phosphorylation. Finally, the DY5 construct can be the target of phosphorylation and transphosphorylate other cell targets such as the wt EGFR. Given that both mAb 528 and 806 can inhibit U87MG.DY5 xenografts but not U87MG.DK xenografts, it suggests that the ability of these antibodies to prevent the phosphorylation of other cellular components is critical to their anti-tumor activity.

[0033] FIGURE 12. **Therapy of U87MG.Δ2-7_{src} xenografts with mAb 806 and Dasatinib alone or in combination.** U87MG.Δ2-7_{src} xenografts were established by

injection of 1×10^6 cells in both flanks of nude BALB/c mice in Therapy commenced when xenografts reached an approximate mean volume of 80 mm^3 . Mice were treated with vehicle (4% DMSO in dH_2O), 1 mg of mAb 806 in PBS, 10 mg/kg^{-1} Dasatinib in 4% DMSO in dH_2O or a combination of both, three times per week for two weeks on the days indicated. Data are expressed as mean tumor volume \pm SE. At day 33 the combination treated group was significantly smaller than the group treated with mAb 806 alone ($p < 0.0076$).

[0034] FIGURE 13. Therapy of U87MG. $\Delta 2-7_{\text{src}}$ xenografts with mAb 806 and Dasatinib alone or in combination. Data from the above experiment were transformed into Kaplan-Meier survival curves and analyzed by Wilcoxon analysis using dual endpoints of moribund or tumor volume $> 1500 \text{ mm}^3$. The combination group survived longer than other groups Log Rank $p < 0.0001$.

DETAILED DESCRIPTION

[0035] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

[0036] Therefore, if appearing herein, the following terms shall have the definitions set out below.

[0037] The term "antibody" describes an immunoglobulin whether natural or partly or wholly synthetically produced. Antibody includes any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, recombinant, humanized, and chimeric antibodies. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. CDR grafted antibodies are also contemplated by this term.

[0038] As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023 and U.S. Patent Nos. 4,816,397 and 4,816,567.

[0039] It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) multivalent antibody fragments (scFv dimers, trimers and/or tetramers (Power and Hudson, J Immunol. Methods 242: 193-204 9 (2000))(ix) bispecific single chain Fv dimers (PCT/US92/09965) and (x) "diabodies", multivalent or multispecific fragments

constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, (1993)).

[0040] An "antibody combining site" is that structural portion of an antibody molecule comprised of light chain or heavy and light chain variable and hypervariable regions that specifically binds antigen.

[0041] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[0042] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v), which portions are preferred for use in the therapeutic methods described herein.

[0043] Antibodies may also be bispecific, wherein one binding domain of the antibody is a specific binding member of the invention, and the other binding domain has a different specificity, *e.g.* to recruit an effector function or the like. Bispecific antibodies of the present invention include wherein one binding domain of the antibody is a specific binding member of the present invention, including a fragment thereof, and the other binding domain is a distinct antibody or fragment thereof, including that of a distinct anti-EGFR antibody, for instance antibody 528 (U.S. Patent No. 4,943,533), the chimeric and humanized 225 antibody (U.S. Patent No. 4,943,533 and WO/9640210), an anti-de2-7 antibody such as DH8.3 (Hills, D. et al (1995) Int. J. Cancer 63(4):537-543), antibody L8A4 and Y10 (Reist, CJ et al (1995) Cancer Res. 55(19):4375-4382; Foulon CF et al. (2000) Cancer Res. 60(16):4453-4460), ICR62 (Modjtahedi H et al (1993) Cell Biophys. Jan-Jun;22(1-3):129-46; Modjtahedi et al (2002) P.A.A.C.R. 55(14):3140-3148, or the antibody of Wikstrand et al (Wikstrand C. et al (1995) Cancer Res. 55(14):3140-3148). The other binding domain may be an antibody that recognizes or targets a particular cell

type, as in a neural or glial cell-specific antibody. In the bispecific antibodies of the present invention the one binding domain of the antibody of the invention may be combined with other binding domains or molecules which recognize particular cell receptors and/or modulate cells in a particular fashion, as for instance an immune modulator (e.g., interleukin(s)), a growth modulator or cytokine (e.g. tumor necrosis factor (TNF), and particularly, the TNF bispecific modality demonstrated in U.S.S.N. 60/355,838 filed February 13, 2002 incorporated herein in its entirety) or a toxin (e.g., ricin) or anti-mitotic or apoptotic agent or factor.

[0044] Fab and F(ab')₂ portions of antibody molecules may be prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptans with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

[0045] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may also contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

[0046] The term "antigen binding domain" describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may bind to a particular part of the antigen only, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an antigen binding

domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

[0047] The terms “mAb806”, “806 antibody”, “monoclonal antibody 806”, “ch806”, “humanized 806” and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to Accordingly, antibodies, including recombinant, chimeric, genetically modified, or alternative antibodies, displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the antibody or its fragments. Also, the terms “mAb806”, “806 antibody”, “monoclonal antibody 806”, “ch806”, “humanized 806” are intended to include within their scope proteins and immunoglobulins specifically recited herein and known to the skilled artisan, publicly disclosed, as well as all substantially homologous analogs and allelic variations. The mAb806 antibody, including its generation, particular activities, amino acid and nucleic acid sequence, antigen binding domains, variable region sequences, are disclosed and known to the skilled artisan, including as provided in WO 02/092771; Luwor RB et al (2001) *Cancer Res* 61:5355-5361; Mishima K et al (2001) *Cancer Res* 61:5349-5354; Johns TG et al (2002) *Int J Cancer* 98:398-408; Jungbluth AA et al (2003) *Proc Natl Acad Sci* 100(2):639-644, each of which is incorporated by reference herein in its entirety.

[0048] It should be appreciated that also within the scope of compositions for use in the methods of the present invention are DNA sequences encoding and/or expressing effective anti-EGFR antibodies, particularly including mAb806 and ch806, which code for anti-EGFR antibodies, antigen binding domains thereof, or active fragments thereof having the same amino acid sequence as the mAb806 antibody as publicly disclosed and known to the skilled artisan, but which are degenerate to the known mAb806 sequence(s). By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid.

[0049] The phrase "src inhibitor" contemplates and includes any modulator which reduces the expression or activity of src, reduces the phosphorylation of the src phosphorylated site, particularly on EGFR, or reduces the signal of the src kinase cascade. A modulator may include a chemical entity, peptide, antibody or other such agent, etc. A modulator may include a kinase inhibitor, phosphatase, etc.

[0050] There are several known small molecule inhibitors of src and some have entered clinical trials, for example dasatinib (BMS354825), AZD-0530, SKI-606, PP1 (4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine), PP2 (4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine), PD166326.

[0051] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[0052] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 20 percent, more preferably by at least 30 percent, still more preferably by at least 50 percent, more preferably by at least 70 percent, more preferably by at least 90 percent, a clinically significant change in the S phase activity of a target cellular mass, or a significant change in the size or dimensions of a target cellular mass or tumor, or other feature of pathology as may attend its presence and activity.

[0053] The antibody or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be

derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0054] The therapeutic antibody or active fragment-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[0055] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition desired or extent of tumor mass being targeted. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

[0056] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "µg" mean microgram, "mg" means milligram, "ul" or "µl" mean microliter, "ml" means milliliter, "l" means liter.

[0057] Thus, both therapeutic and diagnostic applications and methods are provided and raised by the demonstration of the anti tumor activity of anti-EGFR antibody, particularly of mAb806. As suggested earlier and elaborated further on herein, the present invention contemplates pharmaceutical intervention in the cascade of reactions and signaling in which EGFR is implicated, to modulate the tumorigenic capacity associated with EGFR mutations, including kinase domain mutations, both primary and secondary resistant mutations.

[0058] The invention further provides a method of treating EGFR-mediated cancer in a mammal comprising administering to said mammal a src inhibitor and anti-EGFR antibody. In one aspect, the src inhibitor and anti-EGFR antibody are administered simultaneously. In one aspect, the src inhibitor and anti-EGFR antibody are administered simultaneously or serially and repeatedly, before or after traditional chemotherapy.

[0059] The anti-EGFR antibody, particularly mAb806 may be administered in the methods alone or in combination with other anti-EGFR antibodies. MAb806 may also be administered serially or in combination with other anti-EGFR vIII antibodies, including cetuximab, ABX-EGF (panitumumab), DH8.3, L8A4, and or active fragments thereof. The src inhibitor may be administered in the methods alone or in combination with one or more anti-EGFR antibody(ies) and optionally one or more src inhibitor may be administered.

[0060] The anti-EGFR antibody(ies) may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient. A variety of administrative techniques may be utilized, among them parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like. Quantities of the antibody or their active fragments may vary and in particular should be based upon the recommendations and prescription of a qualified physician or veterinarian, including upon consideration of the results and data provided herein.

[0061] The src inhibitor(s) may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient. A variety of administrative techniques may be utilized, among them parenteral techniques such as subcutaneous, intramuscular, intravenous and intraperitoneal injections, catheterizations and the like and/or oral administration or transdermal administration or application. Quantities of the src inhibitor(s) may vary and in particular should be based upon the recommendations and prescription of a qualified physician or veterinarian, including upon consideration of the results and data provided herein. Pharmaceutical compositions which are combinations of one or more anti-EGFR antibody(ies) and one or more src inhibitor(s) may also be prepared suitably for administration.

[0062] Antibodies of the invention may be labeled with a detectable or functional label. Detectable labels include, but are not limited to, radiolabels such as the isotopes ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{121}I , ^{124}I , ^{125}I , ^{131}I , ^{111}In , ^{211}At , ^{198}Au , ^{67}Cu , ^{225}Ac , ^{213}Bi , ^{99}Tc and ^{186}Re , which may be attached to antibodies of the invention using conventional chemistry known in the art of antibody imaging. Labels also include fluorescent labels and labels used conventionally in the art for MRI-CT imaging. They also include enzyme labels such as horseradish peroxidase. Labels further include chemical moieties such as biotin which may be detected via binding to a specific cognate detectable moiety, *e.g.* labeled avidin.

[0063] Functional labels include substances which are designed to be targeted to the site of a tumor to cause destruction of tumor tissue. Such functional labels include cytotoxic drugs such as 5-fluorouracil or ricin and enzymes such as bacterial carboxypeptidase or nitroreductase, which are capable of converting prodrugs into active drugs at the site of a tumor.

[0064] The radiolabeled anti-EGFR antibodies and fragments thereof, are useful in *in vitro* diagnostics techniques and in *in vivo* radioimaging techniques and in radioimmunotherapy. In the instance of *in vivo* imaging, the specific binding members

of the present invention may be conjugated to an imaging agent rather than a radioisotope(s), including but not limited to a magnetic resonance image enhancing agent, wherein for instance an antibody molecule is loaded with a large number of paramagnetic ions through chelating groups. Examples of chelating groups include EDTA, porphyrins, polyamines crown ethers and polyoximes. Examples of paramagnetic ions include gadolinium, iron, manganese, rhenium, europium, lanthanum, holmium and terbium. In a further aspect of the invention, radiolabelled specific binding members, particularly antibodies and fragments thereof, particularly radioimmunoconjugates, are useful in radioimmunotherapy, particularly as radiolabelled antibodies for cancer therapy. In a still further aspect, the radiolabelled specific binding members, particularly antibodies and fragments thereof, are useful in radioimmunoguided surgery techniques, wherein they can identify and indicate the presence and/or location of cancer cells, precancerous cells, tumor cells, and hyperproliferative cells, prior to, during or following surgery to remove such cells.

[0065] Immunoconjugates or antibody fusion proteins of the present invention, wherein the specific binding members, particularly antibodies and fragments thereof, of the present invention are conjugated or attached to other molecules or agents further include, but are not limited to binding members conjugated to a chemical ablation agent, toxin, immunomodulator, cytokine, cytotoxic agent, chemotherapeutic agent or drug.

[0066] Radioimmunotherapy (RAIT) has entered the clinic and demonstrated efficacy using various antibody immunoconjugates. ¹³¹I labeled humanized anti-carcinoembryonic antigen (anti-CEA) antibody hMN-14 has been evaluated in colorectal cancer (Behr TM et al (2002) Cancer 94(4Suppl):1373-81) and the same antibody with ⁹⁰Y label has been assessed in medullary thyroid carcinoma (Stein R et al (2002) Cancer 94(1):51-61). Radioimmunotherapy using monoclonal antibodies has also been assessed and reported for non-Hodgkin's lymphoma and pancreatic cancer (Goldenberg DM (2001) Crit Rev Oncol Hematol 39(1-2):195-201; Gold DV et al (2001) Crit Rev Oncol Hematol 39 (1-2) 147-54). Radioimmunotherapy methods with particular antibodies are also described in U.S. Patent 6,306,393 and 6,331,175. Radioimmunoguided surgery

(RIGS) has also entered the clinic and demonstrated efficacy and usefulness, including using anti-CEA antibodies and antibodies directed against tumor-associated antigens (Kim JC et al (2002) *Int J Cancer* 97(4):542-7; Schneebaum S et al (2001) *World J Surg* 25(12):1495-8; Avital S et al (2000) *Cancer* 89(8):1692-8; McIntosh DG et al (1997) *Cancer Biother Radiopharm* 12 (4):287-94).

[0067] Antibodies of the present invention may be administered to a patient in need of treatment via any suitable route, usually by injection into the bloodstream or CSF, or directly into the site of the tumor. The precise dose will depend upon a number of factors, including whether the antibody is for diagnosis or for treatment, the size and location of the tumor, the precise nature of the antibody (whether whole antibody, fragment, diabody, etc), and the nature of the detectable or functional label attached to the antibody. Where a radionuclide is used for therapy, a suitable maximum single dose is about 45 mCi/m², to a maximum of about 250 mCi/m². Preferable dosage is in the range of 15 to 40 mCi, with a further preferred dosage range of 20 to 30 mCi, or 10 to 30 mCi. Such therapy may require bone marrow or stem cell replacement. A typical antibody dose for either tumor imaging or tumor treatment will be in the range of from 0.5 to 40 mg, preferably from 1 to 4 mg of antibody in F(ab')₂ form. Naked antibodies are preferable administered in doses of 20 to 1000 mg protein per dose, or 20 to 500 mg protein per dose, or 20 to 100 mg protein per dose. This is a dose for a single treatment of an adult patient, which may be proportionally adjusted for children and infants, and also adjusted for other antibody formats in proportion to molecular weight. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician. The dosage and administration of the src inhibitor(s) may be determined and varied by a physician or other individual skilled in the art.

[0068] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1**The Efficacy Of EGFR-Specific Antibodies Is Enhanced Upon
SRC Inactivation Or Inhibition**

[0069] Factors affecting the efficacy of therapeutic monoclonal antibodies (mAbs) directed to the EGFR remain relatively unknown, especially in glioma. The efficacy of two EGFR-specific mAbs was examined (mAb 806 and 528) against U87MG derived glioma xenografts expressing EGFR variants. Using this approach permitted the change of the form of the EGFR while keeping the genetic background constant. These variants included the de2-7 EGFR (or EGFRvIII), a constitutively active mutation of the EGFR expressed in glioma.

[0070] The efficacy of the mAbs correlated with EGFR number, however the most important factor was receptor activation. While U87MG xenografts expressing the de2-7 EGFR responded to therapy, those exhibiting a dead kinase de2-7 EGFR were refractory. A modified de2-7 EGFR that was kinase active but autophosphorylation deficient also responded, suggesting that these mAbs function in de2-7 EGFR expressing xenografts by blocking trans-phosphorylation. Since de2-7 EGFR expressing U87MG xenografts co-express the wt EGFR, efficacy of the mAbs was also tested against NR6 xenografts that expressed the de2-7 EGFR in isolation. While mAb 806 displayed anti-tumor activity against NR6 xenografts, mAb 528 therapy was ineffective, suggesting that mAb 528 mediates its anti-tumor activity by disrupting interactions between the de2-7 and wt EGFR.

[0071] Finally, genetic disruption of Src in U87MG xenografts expressing the de2-7 EGFR dramatically enhanced mAb 806 efficacy. The effective use of EGFR-specific antibodies in glioma will depend on identifying tumors with activated EGFR. The combination of EGFR and Src inhibitors provides a new and effective strategy for the treatment of glioma.

Background

[0072] The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with intrinsic tyrosine kinase activity. Over-expression of the EGFR is observed in numerous epithelial tumors and is often associated with a poorer clinical prognosis (1-3). Over-expression of the EGFR can result from *EGFR* gene amplification, particularly in glioma (4). In glioma, gene amplification is associated with *EGFR* rearrangements with the most common mutation, the de2-7 EGFR (or EGFRvIII), characterized by an in-frame deletion of 801 base pairs spanning exons 2 to 7 of the coding sequence (4-6). This rearrangement results in the deletion of 267 amino acids from the extracellular domain and the insertion of a novel glycine at the fusion site, all of which produces a unique junctional peptide. While the de2-7 EGFR is unable to bind any known ligand, the receptor displays a low level of constitutive activation and is able to enhance the growth of glioma and breast cancer xenografts (7, 8).

[0073] Inhibition of the EGFR is a rational strategy for the development of new cancer therapeutics. Potential therapeutics include monoclonal antibodies (mAbs) directed to the EGFR (e.g. C225, ABX-EGF, EMD 55900) (9-11) and small molecular weight tyrosine kinase inhibitors (TKI's) of the EGFR (e.g. ZD1839, OSI 774) (12). Indeed, some of these therapeutics have been approved for limited clinical use in lung cancer (ZD1839, Iressa) and colon cancer (C225, Erbitux). From these clinical trials it is abundantly clear that not all patients positive for the EGFR respond to these targeted therapeutics (Table 1). Determining factors that cause patients to be susceptible to EGFR therapeutics is an important goal from a patient welfare and economic point of view. Likewise, understanding the nature of resistance to EGFR therapeutics may help identify approaches for overcoming it.

[0074]

TABLE 1

Cellular aspects associated with susceptibility to EGFR therapeutics.

EGFR Inhibitor	Experimental System	Observation	Comment	Ref(s)
PD153035	Multiple cell lines <i>in vitro</i>	Sensitivity correlated	no <i>in vivo</i> data	(32)

		with wt EGFR number		
C225	Renal cell carcinomas <i>in vitro</i>	Only cells containing the <i>VHL</i> gene were sensitive	no <i>in vivo</i> data	(33)
EMD55900 and EMD72000	Multiple cell lines <i>in vitro</i> and xenografts	Sensitivity correlated with wt EGFR number		(34)
SU1195 and ZD1839	Multiple cell lines <i>in vitro</i> and xenografts	More difficult to inhibit the phosphorylation of EGFR in the presence of ErbB2		(35)
mAbR3 and C225	A431 xenografts	Recurrent xenografts following complete regression were often resistant to further therapy	Over-expression of VEGF was a common observation in resistant cell lines	(36)
ZD1839	A431 and NR6M (express the de2-7 EGFR) xenografts	Xenografts expressing the de2-7 EGFR were resistant	NR6M express the de2-7 EGFR in the absence of the wt EGFR, clinically both are co-expressed	(37)
AG1478	Glioma cell lines <i>in vitro</i>	Resistant glioma expresses IGFR-1 which is further up-regulated by AG1478. IGFR-1 effect appears mediated through PI3-K/Akt	Observation restricted to a single cell line <i>in vitro</i>	(38)
CGP59326	BT474 breast and MKN7 gastric cancer cells <i>in vitro</i>	Activation of erbB2/3 heterodimers by heregulin generated resistance	no <i>in vivo</i> data	(39)
ZD1839	Multiple cell lines <i>in vitro</i>	Sensitivity correlated with wt EGFR number. Constitutive active MAPK increased resistance.	no <i>in vivo</i> data	(40)
AG1478	Large cell panel <i>in vitro</i>	Two requirements for sensitivity: high wt EGFR and ability to respond to EGF by entering cell cycle.	no <i>in vivo</i> data	(41)
ZD1839 and PD153035	Multiple cell lines <i>in vitro</i>	Sustained signaling through Akt or Erk may cause resistance.	no <i>in vivo</i> data	(42)
ZD1839	A431 and MDA-468 breast cancer cells <i>in vitro</i>	Sustained signaling through Akt causes resistance. Presence of PTEN increases effectiveness of EGFR therapeutics.	no <i>in vivo</i> data	(43)
ZD1839 and C225	A431 and multiple NSCLC <i>in vitro</i>	No correlation with EGFR number.	no <i>in vivo</i> data	(44)
ZD1839	Patients with NSCLC	Patients with activating mutations in the EGFR kinase domain more likely to respond.	Subsequent data suggests that not all patients with mutations respond	(28, 45)
ZD1839	NR6 fibroblasts and U87MG glioma cells	Cells expressing the de2-7 EGFR were resistant, possibly related to an inability to fully inhibit de2-7	no <i>in vivo</i> data	(46)

		EGFR phosphorylation.		
OSI-774	Panel of glioma cell lines	Cells capable of increasing the mRNA for EGFR in response to therapy are more resistant.		(47)
ZD1839 and OSI-774	Patients with NSCLC	A secondary mutation in EGFR kinase causes resistance		(48)
C225 and ABX	Patients with colorectal cancer	Response correlated with increase in <i>EGFR</i> copy number	Small sample numbers	(26)
OSI-774 and ZD1839	Patients with glioma	Co-expression of EGFRvIII and PTEN is associated with responsiveness		(49)

[0075] Mechanisms causing resistance/susceptibility to EGFR targeted TKI's have been studied extensively, whereas factors affecting the efficacy anti-EGFR antibodies remains relatively unknown (see Table 1). A few generalizations can be drawn from these studies with respect to TKI's. Firstly, the sensitivity of cell lines to inhibition by TKI's correlates with increasing cell surface EGFR (Table 1), suggesting that there is some intrinsic level of EGFR expression required for these inhibitors to function. Secondly, the ability to sustain signalling through the PI3-kinase/Akt pathway following EGFR inactivation reduces the efficacy of TKI's (Table 1). The overwhelming number of these studies has been done *in vitro*, thus it is not known if these observations hold true in the *in vivo* setting. Recently a number of studies have analysed the status of the *EGFR* gene in lung cancer patients treated with Iressa (ZD1839) and found that patients who responded to therapy often had gain of function mutations in the kinase domain (Table 1). Furthermore a secondary kinase mutation that leads to Iressa resistance has also been described (Table 1). Initial studies suggest however that these observations are not general and that the mutations described in lung patients are not observed in other tumor types.

[0076] The limited number of studies using anti-EGFR antibodies makes it difficult to derive any generalizations regarding susceptibility to these agents (Table 1). Apart from the lack of *in vivo* studies, many of these susceptibility studies have been done using cell panels which, given the variation in signalling pathways between cells lines and the presence or absence of other ErbB family members, makes it difficult to identify single

factors associated with EGFR sensitivity or resistance. In order to address some of these issues we tested the *in vivo* susceptibility of the U87MG glioma cell line, which expresses modest levels of the wild type (wt) EGFR, to two EGFR-specific antibodies. We then transfected U87MG cells with a variety of wt and de2-7 EGFR constructs to determine what effect receptor number and activation has on susceptibility to antibody therapy.

[0077] The two antibodies used in this study are mAb 806 and 528. MAb 806 is a novel anti-EGFR specific antibody that was raised against cells expressing the de2-7 EGFR (13). Interestingly, while mAb 806 clearly binds the de2-7 EGFR, it also binds to a subset of the wt EGFR (~10%) expressed on the surface of cells over-expressing the receptor (13). Recent analysis showed that the mAb 806 epitope is only exposed in a conformational form of the EGFR that exists transiently as the receptor moves from its inactive to active state (14). 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 xenografts which express the de2-7 or over-express the wt EGFR with mAb 806 causes significant inhibition of tumor growth (15-17). The 528 antibody was produced and isolated at the same time as the murine version of the C225 antibody (Erbix) and displays very similar properties (18). MAb 528 acts as a ligand antagonist and inhibits the growth of EGFR expressing cells both *in vitro* and *in vivo* when grown as xenografts (18).

Materials And Methods

Cell Lines and Monoclonal Antibodies.

[0078] The U87MG transfected cell lines U87MG.Δ2-7, U87MG.DK, U87MG.wt, U87MG.DY5 and U87MG.DY2 have been described in detail elsewhere (16, 19). The A431 cell line has also been described previously (20). All cell lines were maintained in either DMEM (DMEM/F12; Life Technologies, Inc, Grand Island, NY) or RPMI containing 10% FCS (CSL, Melbourne, Victoria, Australia), 2 mM glutamine (Sigma Chemical Co, St. Louis, MO), and penicillin/streptomycin (Life Technologies, Inc,

Grand Island, NY). In addition, transfected cell lines were maintained in 400mg/ml of Geneticin (Life Technologies, Inc, Melbourne, Victoria, Australia).

[0079] The mAb 806 and 528 were produced and purified in the Biological Production Facility (Ludwig Institute for Cancer Research, Melbourne, Australia). Antibodies to the specific tyrosine phosphorylation sites of the EGFR and a rabbit polyclonal anti-EGFR antibody were obtained from Cell Signaling Technology (Danvers, MA). Src was detected using the mouse monoclonal antibodies v-Src 327 (Oncogene Research Products, CA, USA) or c-Src H-12 (Santa Cruz Biotechnology, Inc, CA, USA). The rabbit polyclonal antibody PY418 (BioSource International, Inc., CA, USA) was used for the detection of phospho-Src. The anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, (Lake Placid, NY). The C13 used for detection of both wild-type and truncated EGFR was obtained from BD Transduction Laboratory (San Diego, Ca).

Generation of U87MG.Δ2-7_{DNSrc} cell line.

[0080] A dominant negative (DN), kinase dead Src construct (K296R/Y528F) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). A Hind III fragment containing the DNSrc was sub-cloned into the pcDNA3.1/Hygro(+) vector obtained from Invitrogen Life Technologies (Carlsbad, CA) and the resulting construct transfected into U87MG.Δ2-7 cells by electroporation. A second cell line transfected with the pcDNA3.1/Hygro vector alone was also generated. Cells were plated out in 1 ml aliquots into 96 well plates, at a density of approximately 2×10^4 cells per well, and incubated at 37°C for 48 hours after which 100 μg/ml hygromycin (Roche Diagnostics, Mannheim, Germany) was added. Once clones were obtained (approximately two weeks) cells were placed back in 400 μg/ml Geneticin as well as the hygromycin.

[0081] Transfected cells were initially screened by FACS analysis to confirm that expression of the de2-7 EGFR had been retained. Clones were then subjected to either whole cell lysis or immunoprecipitation prior to western blotting using Src specific antibodies (v-Src 327, c-Src H-12). Several clones showing dramatically increased levels

of total Src (Src levels are very low in the original cell line) were identified and expanded. The increased Src levels were further confirmed by immunoprecipitating ³⁵S-labelled cell lysates with the v-Src 327 antibody and subjecting the resulting precipitates to SDS-PAGE and quantitative autoradiography. The clone expressing the highest levels of DNSrc was selected and the DNSrc was shown to be phosphorylated at position Y418 suggesting that it is correctly folded.

In vitro growth assays.

[0082] The anti-proliferative effect of mAb 806 and 528 *in vitro* was examined as described in detail previously (18). Briefly, cells were seeded at 1×10^4 cells per well in 24 well plates in media containing 0.5% FCS. After 4 days cells were removed with trypsin and counted using a haemocytometer. Antibodies were used at a final concentration of 100 μ g/ml, a concentration consistent with that obtained within xenografts.

Xenograft models.

[0083] Tumor cells (3×10^6) in 100 μ l of PBS were inoculated s.c. into both flanks of 4-6 week old, female nude mice (Animal Research Centre, Perth, Australia). All studies were conducted using established tumor models as previously reported (15, 16). Treatment commenced once tumors had reached a mean volume of approximately 100 mm³. Tumor volume in mm³ was determined using the formula $(\text{length} \times \text{width}^2)/2$, where length was the longest axis and width being the measurement at right angles to the length. Data are expressed as mean tumor volume \pm SE for each treatment group. All data was analyzed for significance by Student's t test. A minimum of 10 xenografts per group were used in each study.

Immunoblotting.

[0084] Cells were lysed in cold lysis buffer (30 mM HEPES, 150 mM NaCl, 10 mM NaF, 1% Triton X-100, 200 μ M NaO₃V, 0.4% H₂O₂ and the protease inhibitor cocktail set 1 (Calbiochem, San Diego, CA) containing 500 μ M AEBSF, 150 nM Aprotinin, 1 μ M

E-64 protease inhibitor, 0.5 mM EDTA and 1 μ M Leupeptin, pH 7.4). Lysates were immunoprecipitated with the mAb 806 or 528 and the resultant precipitates analyzed by immunoblotting as described by us in detail (21).

Immunofluorescence Microscopy.

[0085] MAbs 806 and 528 were directly labelled with Cyanine 3 (Cy3) dye using the Cy3 Monoclonal Antibody Labeling kit (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England) according to the manufacturer's instructions. Successful labeling of antibody was determined via flow cytometry analysis of binding to U87MG. Δ 2-7 cells. The early endosome specific, anti-mouse Early Endosome Autoantigen 1 (EEA1) monoclonal antibody was purchased from Transduction Laboratories (San Diego, CA, U.S.A.). Cy2 conjugated AffiniPure F(ab')² fragment donkey anti-mouse IgG secondary antibody and unlabeled AffiniPure Fab fragment goat anti-mouse IgG blocking antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). U87MG. Δ 2-7 or NR6. Δ 2-7 cells were grown on 12 mm glass coverslips or 12 mm Biocoat Cell Environments Poly-D-Lysine coverslips (Becton Dickinson labware, Bedford, MA, U.S.A.) in MEM (GibcoBRL Grand Island, NY, U.S.A.) supplemented with 10% FBS, penicillin/streptomycin, and glutamate at 37°C. Antibody binding to cells was carried out in the presence of 0.25% bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO, U.S.A.). Cy3-conjugated mAb 806 and 528 were used at concentrations of 5 μ g/ml and 2 μ g/ml respectively and surface labeling was carried out at 4°C for 20 min under humidified conditions. Cells were washed in ice cold 0.25% Bovine Serum Albumin (BSA)/PBS three times. Internalization of surface bound antibody was initiated by incubation of individual coverslips at 37°C. Following internalization for varying periods of time, individual coverslips were removed from 37°C, washed three times in ice cold BSA/PBS to stop internalization and fixed in 4% PFA for 20 mins at RT. Coverslips were then washed in BSA/PBS prior to washing in double distilled water (DDW) and mounted onto glass slides with fluoromount G mounting medium (Southern Biotechnology, Birmingham, AL, U.S.A.). Samples were analyzed with confocal microscope (Nikon Instech Co., Ltd., Kanagawa, Japan) using appropriate wavelength settings. For co-localization studies, cells were permeabilized with 0.1% triton X-100 for 1 min. Samples

were then washed and incubated with unlabelled goat anti-mouse Fab fragment to block all existing mouse binding sites (i.e. internalized mAb 806 or 528) for 20 min at RT. Samples were then washed in BSA/PBS prior to incubation with anti-EEA1 for 20 min at RT. Cells were finally washed and incubated with Cy2-conjugated secondary donkey anti-mouse F(ab')₂ antibody fragment. DNA vectors for green fluorescence protein (GFP)-tagged Lysosomal Glycoprotein 120 (lgp-120-GFP) was kindly provided by Professor Ira Mellman and Professor from the Department of Cell Biology, Yale University School of Medicine, New Haven, CT, U.S.A. Cells grown in mat-tek glass bottom microwell dishes containing an embedded 14 mm glass coverslip (MatTek Corp. Ashland, MA, U.S.A.), were transfected overnight using LipofectAMINE reagent (Invitrogen™ Life Technologies, Mulgrave, Vic, Australia) following the manufacturer's instructions. Confocal imaging of positively transfected cells, which fluoresced green when excited with 488 nm wavelength light, was undertaken 24 hrs after transfection.

Results

Correlation between in vitro and in vivo sensitivity.

[0086] Many of the studies described in Table 1 have been conducted *in vitro*. Our experience both with mAb and TKI targeted EGFR therapy clearly demonstrates that *in vitro* sensitivity and *in vivo* response do not reliably correlate. Indeed, we recently published an example where two cell lines showing similar sensitivity to the EGFR-specific TKI AG1478 *in vitro*, differed notably in their *in vivo* response to the same agent (22). Using a standard *in vitro* growth inhibition assay previously described for C225, and an antibody concentration consistent with that achieved at the xenograft site, we saw little correlation between antibody inhibition *in vitro* and *in vivo* anti-tumor activity (Table 2). Neither mAb 528 or 806 inhibited the growth of U87MG.Δ2-7 cells *in vitro*, but both antibodies display robust anti-tumor activity *in vivo* that was independent of immune effector function (see Fig. 2). Also, even if one EGFR-targeted antibody showed correlation *in vitro* and *in vivo* in a particular cell line (e.g. mAb 528 in A431 cells and xenografts, Table 2), this did not necessarily imply another EGFR-specific antibody will correlate in the same cell line (e.g. mAb 806 in A431 cells and xenografts, Table 2). This

simple analysis along with our previous observations, clearly demonstrate the limited value of *in vitro* assays in determining sensitivity to EGFR therapeutics.

[0087]

TABLE 2***In vitro and in vivo comparison of sensitivity to EGFR therapeutics***

	CELL LINE			
	U87MG.Δ2-7		A431	
	mAb 528	mAb 806	mAb 528	mAb 806
<i>In Vitro</i>	-	-	+	-
<i>In Vivo</i> *	+	+	++	++

* *In vivo* data for A431 xenografts is from our recent paper (16)

Antibody therapy of U87MG glioma xenografts expressing different forms of the EGFR.

[0088] The parental U87MG cells, which express moderate levels of the wt EGFR, or the same cell line transfected with additional wt EGFR, the de2-7 EGFR or various modified forms of the de2-7 EGFR (Fig. 1) were injected s.c. into nude mice and allowed to establish as tumor xenografts. Treatment with antibody commenced once xenografts had reached approximately 100 mm³. All tumors were treated with 1 mg of mAb 528 or 806 three times per week for 2 weeks. This dose and schedule of antibody treatment was chosen as it elicits a strong anti-tumor response in our standard U87MG.Δ2-7 xenograft model, but is not so efficacious that it would obscure any increased anti-tumor activity that might be seen in other U87MG-derived cell lines containing different variants of the EGFR. As discussed in detail below, the anti-tumor efficacy of mAb 806 and 528 was similar in all the U87MG derived glioma xenografts (Fig. 2).

[0089] **1. Parental cells (U87MG):** Neither antibody inhibited the growth of the U87MG xenografts despite the fact it expresses the EGFR at moderate levels (~ 5 x 10⁴ receptors per cell) (13).

[0090] **2. Cells over-expressing the wt EGFR (U87MG.wt):** Transfection of U87MG cells with the wt EGFR to increase expression (approximately 1×10^6 receptors per cell) did not change the *in vivo* growth rate of the xenografts (Fig. 3A) but did cause the tumors to become sensitive to both antibodies. While this is not surprising for mAb 806, as it preferentially binds to cells over-expressing the wt EGFR, it was somewhat unexpected for mAb 528, as it suggests that even an increase in receptor number in the absence of a phenotypic change can induce a response to antibody therapy. On day 31, when the control group was sacrificed, the inhibition induced by mAb 528 was significant ($p < 0.01$), with xenografts in the vehicle group having a mean tumor volume of 950 mm^3 compared with 450 mm^3 in the mAb 528 treatment group. Analysis of the mAb 806 experiment on day 39 showed that antibody treatment significantly inhibited xenograft growth ($p < 0.001$) with tumor volumes being 960 mm^3 and 470 mm^3 for the PBS and mAb 806 groups respectively.

[0091] **3. Cells expressing the de2-7 EGFR (U87MG. Δ 2-7):** The growth of U87MG xenografts transfected with the constitutively active, but ligand independent, de2-7 EGFR was also inhibited by both antibodies (Fig. 2). Unlike over-expression of the wt EGFR, co-expression of the de2-7 EGFR in the presence of endogenous wt EGFR, generates a significant growth advantage to U87MG xenografts (Fig. 3B). The constitutive phosphorylation of this receptor was confirmed by immunoblotting (Fig. 4). Treatment with mAb 528 significantly inhibited tumor growth ($p < 0.005$) with the vehicle group having an average tumor volume of 1170 mm^3 compared to 510 mm^3 for the mAb 528 group at day 20 post-inoculation. Given that the primary function of mAb 528 has been presumed to be ligand antagonism, its anti-tumor activity against a xenograft expressing the ligand independent de2-7 EGFR was unexpected. Thus, mAb 528 probably disrupts EGFR signalling by other mechanisms apart from its ability to block ligand. Likewise, mAb 806, which only binds the de2-7 EGFR and not the wt EGFR in these cells, must mediate its anti-tumor activity independent of any effect on ligand interaction as it inhibited the growth of de2-7 EGFR expressing xenografts to a similar level as mAb 528. At day 21, when the vehicle group was culled, the control xenografts had a mean tumor volume of 1500 mm^3 compared to a significantly lower 390 mm^2 in the mAb 806 treated

group ($p < 0.0001$). Thus, both antibodies can inhibit glioma xenografts expressing a ligand-independent but constitutively active form of the EGFR.

[0092] 4. Cells expressing a dead kinase version of the de2-7 EGFR (U87MG.DK):

U87MG cells transfected with a dead kinase (DK) version of the de2-7 EGFR grew as xenografts at a rate similar to parental cells (Fig. 3B) and were not significantly inhibited by either antibody (Fig. 2). This receptor lacks phosphorylation at the major sites associated with signalling but remains phosphorylated at sites associated with receptor internalization and degradation (Fig. 4). Binding of both antibodies to these cells is similar to that seen in de2-7 EGFR expressing cells both *in vitro* and *in vivo* (16). Furthermore, since the DK variant of the de2-7 EGFR only contains a single intracellular point mutation, the affinity of mAb 806 and 528, which bind the extracellular domain, should not be altered. This result demonstrates that any immune effector function mediated by these antibodies *in vivo* is insufficient to initiate an anti-tumor response. Furthermore, it shows that the anti-tumor activity of anti-EGFR antibodies require a receptor with a functional kinase domain.

[0093] 5. Cells expressing a version of the de2-7 EGFR with deletion of 2 major sites for autophosphorylation (U87MG.DY2):

U87MG xenografts expressing a de2-7 EGFR construct unable to autophosphorylate at two major autophosphorylation sites (tyrosine 1068 and 1173 changed to phenylalanine) were significantly inhibited by both antibodies when grown as tumor xenografts ($p < 0.01$ and 0.006 for mAb 528 and 806 respectively) (Fig. 2). This observation, combined with the lack of activity seen against the U87MG.DK xenografts, suggests that the kinase activity, as opposed to autophosphorylation, correlates with responsiveness to antibody therapy.

[0094] 6. Cells expressing a version of the de2-7 EGFR incapable of

autophosphorylation (U87MG.DY5): U87MG cells expressing a de2-7 EGFR construct unable to autophosphorylate at all 5 major autophosphorylation sites associated with signaling (tyrosine 1173, 1148, 1086, 1068 and 992 changed to phenylalanine) were grown as tumor xenografts. This receptor lacks phosphorylation at the major sites

associated with signaling but remains phosphorylated at sites associated with receptor internalization and degradation (Fig. 4). Consistent with the result obtained with DY2 xenografts, both antibodies significantly inhibited the growth of xenografts expressing the DY5 de2-7 EGFR construct ($p < 0.0001$ for both antibodies) (Fig. 2). Given this somewhat unexpected result, we repeated this experiment with both antibodies, at a lower dose (0.5 versus 1 mg per injection), and once again obtained significant inhibition of tumor growth in both cases (data not shown). Since the DY5 form of the de2-7 EGFR is incapable of directly binding adapter molecules critical for downstream signaling, it suggests that an active kinase domain rather than the interaction with these molecules, is a critical feature that leads to responsiveness to EGFR-specific antibodies.

Treatment of U87MG Xenografts Expressing High Levels of the de2-7 EGFR.

[0095] The data in Fig. 2 suggests that the more dependent a xenograft becomes to EGFR signaling the more likely it is to respond to EGFR-specific antibody therapy. Therefore, using FACS sorting we isolated the cells expressing very high levels of the de2-7 EGFR (U87MG. Δ 2-7_{high}) (Fig. 5A). U87MG. Δ 2-7_{high} xenografts grew faster than the original U87MG. Δ 2-7 xenografts (Fig. 5B), suggesting that the rapid growth of these xenografts is reliant on the high levels of the de2-7 EGFR. The levels of de2-7 EGFR expression were retained *in vivo* as determined by immunoblotting of xenograft lysates (Fig. 5C).

Treatment with mAb 806 or mAb 528 caused significant inhibition of U87MG. Δ 2-7_{high} xenografts that was greater than that observed for any other of the U87MG derived cell lines (Fig. 5D). On day 18, when the control group was sacrificed for ethical reasons, the mean tumor volume was 1760, 90 and 90 mm³ for the vehicle, mAb 806 and mAb 528 groups respectively ($p < 0.001$). Significantly, while there were no complete regressions in any of the previous U87MG-derived therapy studies (Fig. 2), 40% of the mAb 806 and 20% mAb 528 treated U87MG. Δ 2-7_{high} xenografts completely regressed. One of the mAb 806 tumors recurred at day 46 post-inoculation while other tumors had not recurred by day 126 when the mice were sacrificed. Thus, xenografts driven by the over-expression of a constitutively active form of the EGFR are more sensitive to EGFR-specific antibodies.

mAb 806 and 528 Therapy of Established NR6 Derived Xenografts.

[0096] The NR6 murine fibroblastic cell line does not endogenously express any members of the ErbB family (23), an observation we confirmed by FACS for EGFR, ErbB2 and ErbB3 (data not shown). These cells were then stably transfected with human de2-7 EGFR (NR6.Δ2-7). Since all the U87MG derived cell lines used to test the efficacy of mAb 806 and 528 against the de2-7 EGFR also co-express the wt EGFR we assessed their therapeutic efficacy in mice with established NR6.Δ2-7 xenografts. mAb 806 treatment resulted in a reduction in overall tumor growth rate compared to treatment with vehicle that was highly significant at day 42 post-inoculation ($P < 0.003$) (Fig. 6). The average tumor volume on the final day of therapy (day 39) was 1520 and 670 mm³ for the vehicle and mAb 806 treatment groups respectively (Fig. 6A).

[0097] Mice bearing established NR6.Δ2-7 xenografts were also treated with mAb 528. On day 56 post-inoculation, when animals were killed for ethical reasons, the size of tumors treated with mAb 528 did not differ from that of vehicle treated xenografts (Fig. 6B). We conducted a second therapy experiment with mAb 528 using a slightly varied protocol whereby mice received antibody twice per week for three weeks. Once again mAb 528 failed to inhibit the growth of established NR6.Δ2-7 xenografts under these conditions (Fig. 6C). Thus, unlike mAb 806, mAb 528 is unable to inhibit xenografts expressing the de2-7 EGFR in the absence of the wt EGFR.

Src Activity Modulates the Responsiveness of de2-7 EGFR Expressing Xenografts to Antibody Therapy.

[0098] Since mAb 806 and 528 inhibit xenografts expressing the DY5 version of the de2-7 EGFR and because neither antibody decreases de2-7 EGFR phosphorylation as a single agent *in vivo* (16), it is likely that these antibodies mediate their anti-tumor activity by disrupting the trans-phosphorylation of a target downstream of the de2-7 EGFR. Our observations with the NR6.Δ2-7 xenografts suggest that the anti-tumor activity of mAb 528 is dependent on the co-expression of the de2-7 EGFR with another member of the ErbB family, whereas mAb 806 activity is independent of this interaction. Therefore, we

examined if the de2-7 EGFR could interact with Src, as is the case for the wt EGFR, and if this potential interaction is related to mAb 806 efficacy.

[0099] Activation of the wt EGFR leads to the transient activation of Src kinase. In a synergistic manner, activation of Src leads to phosphorylation tyrosine 845 (Y845) on the EGFR, which is not an auto-phosphorylation site rather a target for Src phosphorylation (24). Using an antibody specific to Y845 we examined the phosphorylation of Y845 in the de2-7 EGFR. When expressed in U87MG glioma cells the de2-7 EGFR showed robust phosphorylation of Y845 (Fig. 7A). Phosphorylation at Y845 was rapidly blocked by incubating cells with PP1 and PP2, inhibitors of the Src-family kinases, while the autophosphorylation site at Y1173 was relatively unaffected (Fig 7A).

[00100] Given that the de2-7 EGFR appears to be a target for Src kinase phosphorylation in a manner analogous to that of the wt EGFR, we sought to determine if this interaction was critical to mAb 806 activity. Initially we constructed a de2-7 EGFR containing a Y845F substitution, however this protein showed reduced phosphorylation at multiple sites (Johns, *unpublished observations*) and was therefore considered unsuitable for these studies. Thus, as described in the materials and methods, we developed a U87MG cell line co-expressing the de2-7 EGFR and a DNSrc (U87MG.Δ2-7_{DNSrc}). U87MG.Δ2-7_{DNSrc} xenografts grew as tumor xenografts in nude mice but at a rate slower than U87MG.Δ2-7 transfected with a vector control (Fig. 7B). Treatment of U87MG.Δ2-7_{DNSrc} with mAb 806 resulted in robust inhibition of tumor growth (Fig. 7C). At day 34 post-inoculation, the average xenograft volume was 1220 mm³ in the vehicle group compared with 100 mm³ in the mAb 806 treated group (p < 0.001) (Fig 7C). Furthermore, 60% of all U87MG.Δ2-7_{DNSrc} xenografts in the mAb 806 treated group completely regressed and had not recurred by day 50 post-inoculation. Thus, inhibition of Src signaling increases the efficacy of mAb 806 therapy (Fig 7C cf Fig 2).

[00101] *Internalization of mAb 806 in U87MG.Δ2-7 cells.* The intracellular trafficking of mAb 806 following binding to de2-7 EGFR expressed in U87MG.Δ2-7 cells, was investigated by confocal microscopy. Following incubation of mAb 806-Cy3

at 4°C and prior to chase at 37°C, mAb 806 bound to de2-7 EGFR was located along the plasma membrane (Fig. 8A; 0 min, mAb 806-Cy3). Following incubation at 37°C, mAb 806 (Fig. 8A; mAb 806-Cy3) was observed to translocate to small, punctate, cytoplasmic vesicles. Subsequent immunostaining with anti-early endosome autoantigen 1 (EEA1), which identifies early endosomes (Fig. 8A; EEA1), showed partial co-localization with mAb 806 as visualized by the presence of yellow fluorescence (Fig. 8A; Merge). Following 60 mins chase at 37°C, the co-localization was minimal (Fig. 8A; Merge, 60 min), suggesting that the majority of antibody has moved out of early endocytic compartments. These observations indicate that mAb 806 localizes to early endocytic compartments immediately following internalization before moving to an alternative location later in its intracellular trafficking cycle.

[00102] Lysosomal localization of mAb 806 following binding and internalization of de2-7 EGFR in U87MG.Δ2-7 cells was accomplished via co-localization analysis in cells transiently transfected with lgp-120-GFP (Fig. 8B). Cells positively transfected for lgp-120-GFP displayed cytoplasmic peri-nuclear green fluorescence consistent with localization to lysosomal compartments as expected (Fig. 8B; lgp-120-GFP). Prior to induction of internalization, mAb 806-Cy3 was only detected on the cell surface (Fig. 8B; 0 min, mAb 806-Cy3), and did not co-localized with lgp-120-GFP (Fig. 8B; 0 min, merge). Following warming to 37°C for 30 min, small intracellular vesicular structures corresponding to internalized mAb 806 were observed (Fig 8B; 30 min, mAb 806-Cy3). Some of these structures co-localized with lgp-120-GFP, however the majority of red and green signal remained segregated (Fig. 8B; 30 min, merge). Longer incubation at 37°C for 60 and 120 mins resulted in increased co-localization of internalized mAb 806-Cy3 and lgp-120-GFP (Fig 8B; 60-120 min, merge). These observations are consistent with the hypothesis that mAb 806 initially traverses through early endocytic compartment, but after longer periods moves into lysosomal compartments where it accumulates.

[00103] The internalization of mAb 806 following binding to the de2-7 EGFR expressed on U87MG.Δ2-7 cells was also analyzed by electron microscopy. Following 5 mins incubation at 37°C, gold particles, corresponding to mAb 806, were observed in

structures resembling clathrin-coated pits (Fig. 9A and B). Gold particles were also detected in free clathrin-coated vesicles located within the cytoplasm (Fig. 9C). No gold particles were observed in structures resembling caveolae (Fig. 9D). Following 10 min of chase at 37°C, mAb 806 localized to large tubular-vesicular structures resembling early endocytic compartments (Fig 9E). Longer chase periods of 30 mins resulted in antibody localization in structures resembling multivesicular bodies (Fig. 9F). These observations are consistent with the immuno-fluorescence microscopy data that indicated co-localization of mAb 806 with Igp-120 between 30 and 60 mins.

Internalization of MAb 806 and 528 in NR6.Δ2-7 Cells.

[00104] Given the differences in therapeutic efficacy of mAbs 806 and 528 against NR6.Δ2-7 xenografts, the internalization characteristics of each antibody was investigated in this cell line. Furthermore, since NR6.Δ2-7 cells do not express any endogenous members of the ErbB family, this cell line can determine if the presence of wt EGFR is required for internalization of these antibodies. Cells incubated with mAb 806-Cy3 at 4°C showed membrane staining with no intracellular fluorescence as expected (Fig. 10; mAb 806, 0 min). In contrast to U87MG.Δ2-7 cells (Fig. 8), membrane staining was not uniform. More intense staining was associated with membrane junctions between cells (Fig. 10; mAb 806, 0 min) and focal adhesions (Fig. 10; mAb 806, 0 min). Some cells showed very little membrane staining (Fig. 10; mAb 806, 0 min). Following induction of internalization by raising the temperature to 37°C, characteristic intracellular punctate vesicular structures were observed. These accumulated in a peri-nuclear pattern (Fig. 10; mAb 528 15-60 min) consistent with rapid lysosomal localization. Initial localization (Fig. 10; mAb 528, 0 min) and subsequent internalization (Fig. 10; mAb 528, 1-60 min) of mAb 528 was identical to that of mAb 806. Thus both antibodies were rapidly internalized to the lysosomal compartment following binding to the de2-7 EGFR even in the absence of the wt EGFR.

Discussion

[00105] *mAb 528.* Many, but not all, previous studies have suggested that EGFR number on the cell surface is one factor that influences the efficacy of EGFR targeted

therapeutics, especially TKI's (Table 1). However, these experiments have always compared anti-tumor activity using different cell lines and thus are not controlled with respect to genetic background, the presence of other ErbB family members and the occurrence of other functional receptors/kinases capable of modulating the EGFR signaling pathway. Furthermore, many of these studies have been conducted *in vitro*, which we have shown does not correlate with *in vivo* activity. Increasing the wt EGFR number 10-fold converts U87MG glioma xenografts from mAb 528 resistant to antibody responsive. Since the increase in wt EGFR number did not alter the growth rate of the U87MG xenografts, the advent of anti-tumor activity was not simply the result of mAb 528 inhibiting an induced growth advantage. The presence of more wt EGFR within U87MG.wtEGFR xenografts would almost certainly lead to increased antibody localization at the tumor site. Given that mAb 528 possess low, but measurable, immune effector function (25), the increased level of antibody at the tumor site may result in increased complement deposition and recruitment of immune cells that contribute to inhibition of tumor growth. However, a role for immune effector function in initiating the anti-tumor activity of mAb 528 seems unlikely given our data with the U87MG.DK xenografts. These xenografts have as many mAb 528 binding sites as the U87MG.wtEGFR xenografts but are not inhibited by the antibody. One intriguing possibility is that over-expression of the wt EGFR leads to ligand independent EGFR signaling (the parental U87MG appear not to have a strong autocrine-ligand loop), which in turn causes the cells to become more dependent on the EGFR signaling system. Thus, U87MG.wtEGFR xenografts respond to mAb 528 therapy because, unlike the parental cell line, the EGFR signaling pathway is active and functional. Therefore, over-expression of the wt EGFR is a surrogate marker of cells dependence on EGFR signaling and therefore such cells are more likely, but not guaranteed, to respond to EGFR therapeutics (26).

[00106] It has been presumed that the anti-tumor activity of antibodies such as mAb 528 is predominantly mediated by their ability to antagonize ligand activation of the EGFR. Given that mAb 528 inhibited the growth U87MG.wtEGFR xenografts in the absence of significant ligand expression suggests that other mechanisms may contribute

to the anti-tumor effect. Furthermore, mAb 528 displayed significant efficacy against xenografts expressing the ligand independent de2-7 EGFR. This anti-tumor activity could not directly result from mAb 528 binding the endogenous wt EGFR co-expressed in these xenografts, as it did not inhibit the growth of parental U87MG or U87MG.DK xenografts, both of which express identical levels of the wt EGFR. Excluding immune effector function, alternate anti-tumor mechanisms could include receptor down-regulation, induction of inappropriate signaling, translocation of the receptor to unsuitable membrane domains and interference with receptor dimerization and/or oligomerization. Indeed, some TKI's directed to the EGFR not only function by inhibiting kinase activity but induce inactive dimers capable of "mopping" up excess ligand, an unanticipated anti-tumor mechanism (27).

[00107] Interestingly, a recent immunohistochemistry study analyzing EGFR expression in colon patients showing differential response to C225, reported that several patients "negative" for EGFR had clinical responses to this EGFR-specific antibody (26). Presumably these patients have levels of EGFR below the detection sensitivity of immunohistochemistry yet the EGFR present is activated and contributes to tumor growth/survival. This observation suggests that EGFR activation is at least as important, if not more so, than simply the level of EGFR expression. Our data showing that mAb 528 did not inhibit the growth of U87MG xenografts expressing a dead kinase version of this truncated receptor (U87MG.DK) supports the view that the efficacy of EGFR-specific antibodies is intimately associated with kinase active receptors. As suggested above, EGFR over-expression represents one mechanism by which this activation can occur; the expression of a constitutively active mutant such as the de2-7 EGFR denotes another. This continuous activation of the EGFR causes cells to become "addicted" to EGFR signaling, which in turn makes them susceptible to anti-EGFR therapy. This concept is analogous to the situation in lung cancer patients, where most patients who respond to EGFR-specific TKI's carry activating mutations in the EGFR kinase domain (28).

[00108] The ability of mAb 528 to inhibit the growth of U87MG.DY2 or DY5 xenografts highlights the significance of an active kinase domain as opposed to autophosphorylation as a determinant of efficacy. Thus, it is an active kinase that determines the response to antibody therapy, not the direct interaction of phosphorylated tyrosine's with adapter or signaling molecules. One corollary to this result is that mAb 528 seemingly inhibits the growth of U87MG.Δ2-7/DY2/DY5 xenografts by preventing the trans-phosphorylation of a downstream target (Fig. 11). Since all these U87MG-derived cell lines co-express the wt EGFR, and given that we recently demonstrated that the de2-7 EGFR can form dimers and phosphorylate the wt EGFR (29), the wt EGFR is a likely candidate for this secondary target. This proposition is supported by the fact that NR6 cells expressing the de2-7 EGFR in the absence of the wt EGFR were completely refractory to the anti-tumor effects of mAb 528. Taken together these studies suggest that, along with its ligand blocking properties, mAb 528 functions in part by preventing the homo-dimerization of the over-expressed wt EGFR and hetero-dimerization between the wt and de2-7 EGFR. Interestingly, the structure of C225 (an antibody very similar to mAb 528) in complex with the EGFR suggests that apart from ligand blockade, this antibody may prevent EGFR dimerization by partially inhibiting EGFR untethering (30).

[00109] *mAb 806*. Responsiveness of U87MG-derived cell lines *in vivo* to mAb 806 completely mirrored that observed with mAb 528, indicating that many of the above principles apply although there are some important differences. This study confirms and extends our previous studies demonstrating that mAb 806 reactivity is associated with EGFR activation (16). Unlike mAb 528, and all current antibodies in clinical evaluation, mAb 806 does not target normal tissue such as the liver, as EGFR activation is extremely low or non-detectable in organs such as the liver. A myriad of factors can stimulate EGFR activation within tumors (see (31) for a review). We have confirmed that at least three of these, EGFR over-expression (15), mutation (17) and presence of an autocrine loop (Johns *et al*, *in preparation*) can lead to mAb 806 reactivity. The association of wt EGFR over-expression for mAb 806 anti-tumor activity is intimately related to its unique specificity as over-expression increases the transient, untethered form of the EGFR recognized by mAb 806, through multiple mechanisms such as ligand independent

activation and alterations in EGFR glycosylation (21). Given that the work described here, along with the clinical data obtained with EGFR-specific TKI's, suggests that EGFR inhibitors are most effective against tumors with an activated EGFR, the unique ability of mAb 806 to specifically recognize activated forms of the EGFR makes it an advantageous therapeutic.

[00110] Molecular modeling suggests that mAb 806 binding would prevent the formation of active wt EGFR dimers (14), a hypothesis we have confirmed by solving the crystal structure of mAb 806 in complex with its epitope (Johns *et al. in preparation*). Despite this mAb 806 does not significantly inhibit the phosphorylation of the de2-7 or wt EGFR in xenograft models (16), strongly suggesting that any proposed mechanism of action for mAb 806 includes more than blockade of auto-phosphorylation. Furthermore, known down-stream targets of EGFR signaling such as Akt and MAPK, are also not inhibited by mAb 806 (T.G. Johns, unpublished observations). Consistent with this hypothesis, mAb 806 displayed robust anti-tumor activity against U87MG.DY2/DY5 xenografts, two models where autophosphorylation is not pertinent. The lack of mAb 806 efficacy against U87MG.DK xenografts, emphasizes that the presence of an active kinase and trans-phosphorylation events (Fig. 11) are critical factors leading to sensitivity. In contrast to mAb 528, mAb 806 was able to inhibit the growth of NR6 cells expressing the de2-7 EGFR in the absence of other ErbB family members. This result indicates that mAb 806 potentially disrupts other targets of de2-7 EGFR trans-phosphorylation, distinct from the wt EGFR. Interestingly, there was no obvious difference in the internalization and intracellular tracking of mAb 806 and 528 following binding of either antibody to surface de2-7 EGFR in NR6 cells, suggesting antibody trafficking did not contribute to the difference in efficacy in this xenograft model.

[00111] We report here for the first time that Y845 is phosphorylated on the de2-7 EGFR in a Src-dependent manner. Thus, we examined whether the interaction between the de2-7 EGFR and Src was a potential target of mAb 806 activity. If mAb 806 mediated part of its anti-tumor activity by inhibiting this interaction, then genetically disrupting this interaction using a DNSrc should have reduced the efficacy of mAb 806.

In contrast to this possibility, the presence of a DNSrc dramatically enhanced the anti-tumor activity of mAb 806. This suggests that Src has a role in limiting the efficacy of EGFR therapeutics and provides a rationale for using Src and EGFR inhibitors in combination.

Conclusion

[00112] These studies demonstrate the relevance of *in vivo* studies for analyzing the sensitivity of cell lines to EGFR therapeutics. Unlike previous studies we were able to conduct most of our analysis in the same genetic background, making the predominant variable the nature of the EGFR. Using this approach we conclusively showed the significance of receptor number to efficacy. While EGFR number is related to EGFR therapeutic susceptibility, this factor alone is not enough as the receptor also needs to contain a functional kinase. Indeed, while somewhat intuitive, this work shows formally that “forcing” a cell line to use EGFR signaling, either by over-expression of the wt EGFR or expression of a constitutive active mutant, can switch it from non-responsive to responsive. Thus, the EGFR must not only be present at the cell surface, it must be significantly contributing the growth and survival of the cell. Therefore, strategies for selecting patients who will respond to EGFR therapeutics should be directed to identifying tumors highly dependent on the EGFR, not merely the presence or absence of receptor protein. This task may be relatively straight forward in some cases such as when the de2-7 EGFR, *EGFR* gene amplification or kinase activating mutants are present, but is clearly more difficult in cases where the wt EGFR is genetically normal. In these cases the complex interplay of multiple receptor kinases makes it difficult to identify those tumors truly dependent on EGFR signaling. Long term, detailed expression profiling of yet to be identified target genes unique to each receptor kinase may be the only viable approach to addressing this problem.

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EXAMPLE 2**Animal Therapy Study of Src Inhibitor Dasatinib and mAb806 Therapy**

[00114] Animal therapy studies were performed to assess in vivo effects of the anti-EGFR antibody mAb806 alone or in combination with the src inhibitor dasatinib. Eight week old female Balb/C nu nu mice were injected with 1×10^6 U87MG. Δ 2-7_{SRC} cells (per tumour site) subcutaneously. The U87MG. Δ 2-7_{SRC} cells express an activated Src (Y529F mutation) and the Δ 2-7 mutant EGFR. Two tumours were generated per mouse by injection of these cells to each of the right and left flanks. Treatment was commenced when mean tumour size reached approximately 80mm³. Mice were treated three times per week for two weeks in four treatment groups, consisting of 4-5 mice per group. Treatment groups were as follows: (1) control - 100 μ l of diluents 4% DMSO/dH₂O; (2) dasatinib – 10mg/kg of drug dissolved in diluents; (3) mAb806 -1mg; (4) mAb806 1mg and dasatinib 10mg/kg.

[00115] *Antibodies.* Src was detected using the mouse monoclonal antibodies v-Src 327 (Oncogene Research Products, CA, USA) or c-Src H-12 (Santa Cruz Biotechnology, Inc, CA, USA). The rabbit polyclonal antibody PY418 (BioSource International, Inc., CA, USA) was used for the detection of phospho-Src.

Construction of U87MG. Δ 2-7_{src} cell line. An activated Src construct (Y529F mutation) was obtained from Upstate Technologies (Lake Placid, NY, USA). A PmeI fragment containing the activated Src c-DNA was subcloned into the pcDNA3.1/Hygro(+) vector obtained from Invitrogen Life Technologies (Carlsbad, CA), prior to the transfection of U87MG. Δ 2-7 by electroporation. Cells were plated out in 1 ml aliquots into 96 well plates, at a density of approximately 2×10^4 cells per well, and incubated at 37°C for 48 hours after which 100 μ g/ml hygromycin (Roche Diagnostics, Mannheim, Germany) and 400 μ g/ml geneticin (Invitrogen Life Technologies, Carlsbad, CA) was added.

[00116] Transfected cells were initially screened by FACS analysis to confirm that expression of the Δ 2-7 EGFR had been retained. Clones were then subjected to either whole cell lysis or immunoprecipitation prior to western blotting using Src specific

antibodies (v-Src 327, PY418). Several clones showing dramatically increased levels of both total and phosphorylated Src (Src levels are very low in the original cell line) were identified and expanded.

[00117] *Xenograft models.* Tumor cells (1×10^6) in 100 μ l of PBS were inoculated s.c. into both flanks of 8 week old, female nude mice (Animal Research Centre, Perth, Australia). All studies were conducted using established tumor models as previously. Treatment commenced once tumors had reached a mean volume a mean volume of approximately 80 mm³. Tumor volume in mm³ was determined using the formula $(\text{length} \times \text{width}^2)/2$, where length was the longest axis and width being the measurement at right angles to the length. Data are expressed as mean tumor volume \pm SE for each treatment group (Figure 12). All data was analyzed for significance by Student's t test. Data was also transformed into Kaplan-Meier survival curves and analyzed by Wilcoxon analysis using dual endpoints of moribund or tumor volume > 1500 mm³ (Figure 13).

[00118] At day 33 the combination mAb806 and dasatinib treated group tumour growth was significantly smaller than the group treated with mAb 806 alone ($p < 0.0076$) (Figure 12). Data from the tumour growth experiment were transformed into Kaplan-Meier survival curves and analyzed by Wilcoxon analysis using dual endpoints of moribund or tumor volume > 1500 mm³ (Figure 13). The combination mAb806 and dasatinib treated group survived longer than all other groups (Log Rank $p < 0.0001$).

[00119] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[00120] Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

CLAIMS

1. A method of treating cancer in a mammal comprising administering to said mammal the src inhibitor dasatinib (BMS354825) and an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, wherein said src inhibitor dasatinib (BMS354825) and said anti-EGFR antibody or antigen-binding fragment thereof are administered simultaneously, in combination, or one after another in series.
2. The method of claim 1 wherein the anti-EGFR antibody is mAb806 or an active fragment thereof.
3. The method of claim 1 wherein said cancer is selected from the group consisting of glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, bladder cancer, colon cancer, melanomas, gastric cancer, pancreatic cancer, brain cancers, and blood cancers.
4. A method for blocking or reducing tumor growth of an EGFR-mediated cancer in a mammal comprising administering to said mammal the src inhibitor dasatinib (BMS354825) and anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and not is detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, wherein said src inhibitor dasatinib (BMS354825) and anti-EGFR antibody are administered simultaneously, in combination, or one after another in series.
5. The method of claim 4, wherein the anti-EGFR antibody is mAb806 or an active fragment thereof.

6. The method of claim 4, wherein said EGFR-mediated cancer is selected from the group consisting of glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder cancer.

7. A method of enhancing the effectiveness or activity of an anti-EGFR antibody or an antigen-binding fragment thereof in a mammal comprising administering to said mammal a combination of the anti-EGFR antibody or antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, and the src inhibitor dasatinib (BMS354825).

8. The method of claim 7, wherein the anti-EGFR antibody is mAb806 or an active fragment thereof.

9. A pharmaceutical composition comprising an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFRvIII/ Δ 2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, and the src inhibitor dasatinib in a pharmaceutically acceptable carrier or diluent.

10. The composition of claim 9, wherein the anti-EGFR antibody is mAb806 or an active fragment thereof.

11. The method of any one of claims 1 to 3, wherein the cancer is an EGFR mediated cancer.

12. The method of claim 7 or 8, wherein the mammal has an EGFR mediated cancer.
13. The method of any one of claims 4 to 6, 11 or 12, wherein the EGFR mediated cancer is characterized by EGFR over-expression of an EGFR mutation.
14. The method of claim 13, wherein the EGFR mutation is a de2-7 EGFR mutation.
15. Use of the src inhibitor dasatinib (BMS354825) or an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFR^{vIII}/Δ2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, for the manufacture of a medicament or medicaments for the treatment of cancer in a mammal, wherein in use of said medicament(s) said src inhibitor dasatinib (BMS3454825) and said anti-EGFR antibody or antigen-binding fragment thereof are administered simultaneously, in combination, or one after another in series.
16. Use of the src inhibitor dasatinib (BMS354825) or an anti-EGFR antibody or an antigen-binding fragment thereof, which recognizes an EGFR epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal cells and is present or available in the EGFR^{vIII}/Δ2-7 EGFR mutant, but is distinct from the mutant's junctional peptide LEEKKGNYVVTDH, for the manufacture of a medicament or medicaments for blocking or reducing tumor growth of an EGFR-mediated cancer in a mammal, wherein in use of said medicament(s) said src inhibitor dasatinib (BMS3454825) and said anti-EGFR antibody or antigen-binding fragment thereof are administered simultaneously, in combination, or one after another in series.

Ludwig Institute for Cancer Research

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

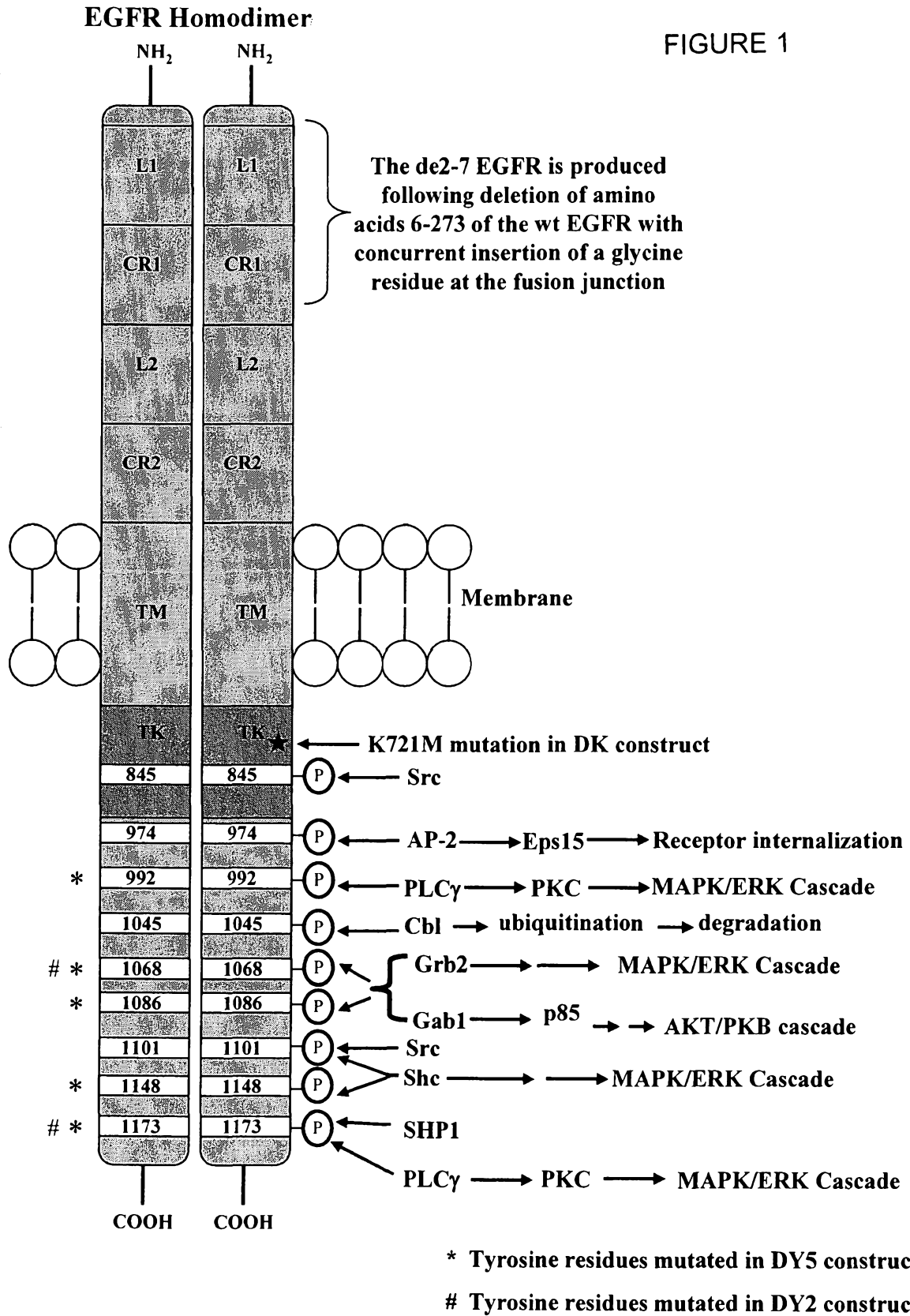


FIGURE 2A

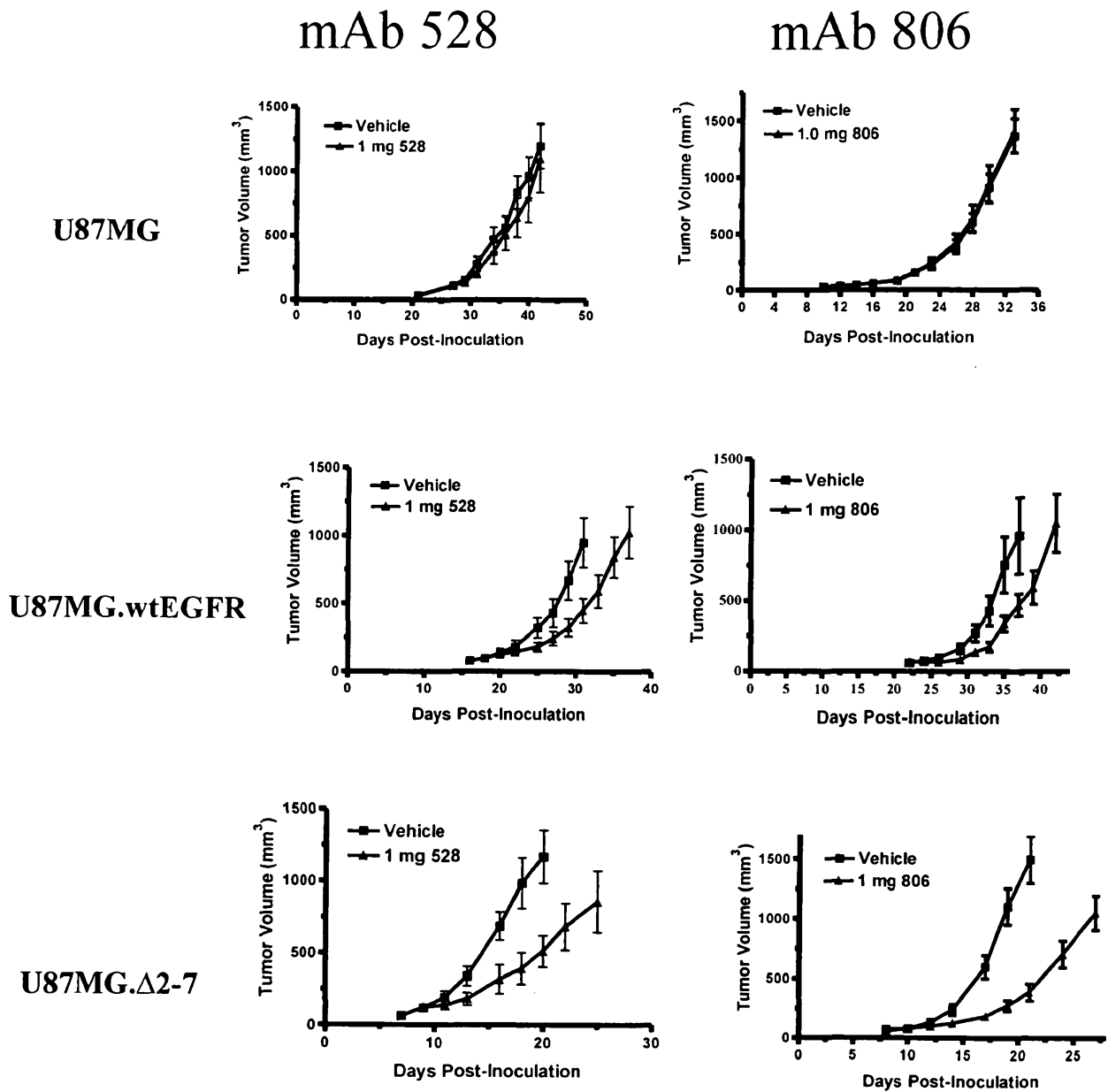
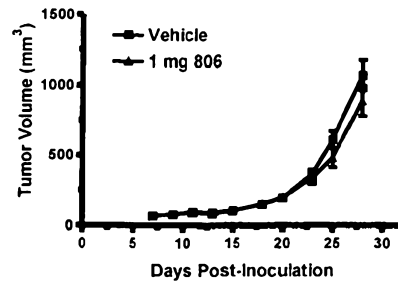
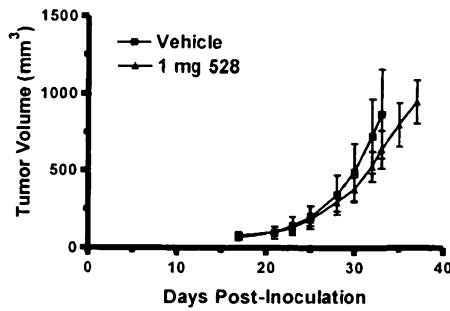


FIGURE 2B

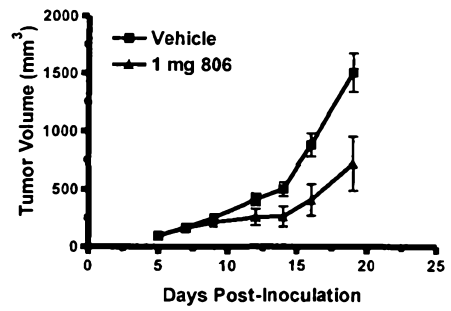
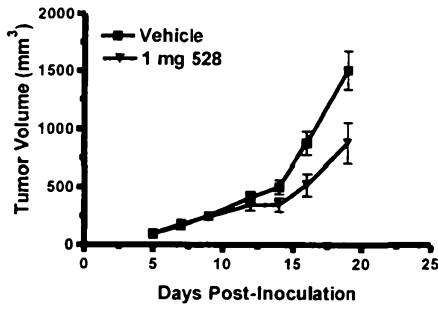
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mAb 806

U87MG.DK



U87MG.DY2



U87MG.DY5

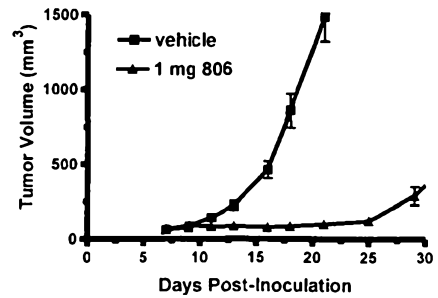
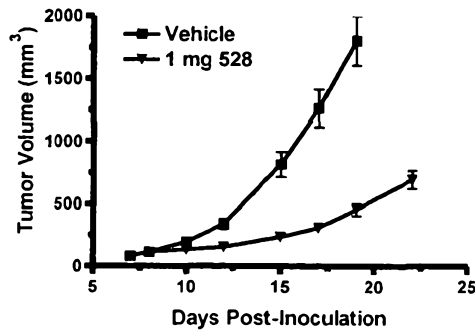


FIGURE 3

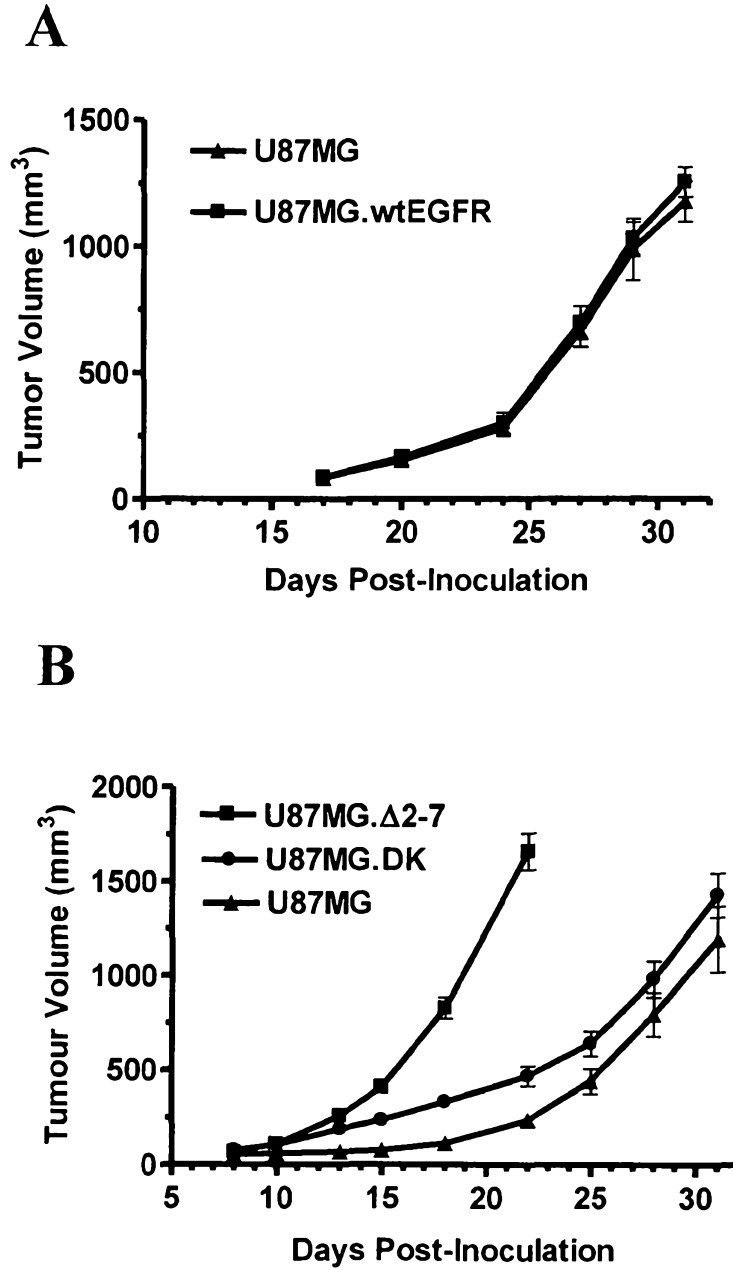
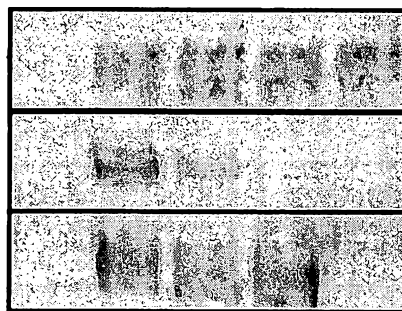


FIGURE 4

A

IP: IgG2b 528 806 528 528



IB: pTyr 1045

IB: pTyr 1173

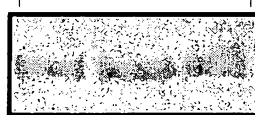
IB: EGFR

Cell Line: Δ 2-7 Δ 2-7 Δ 2-7 DK DY5

In vitro

B

IP: 528



IB: mAb 806

Cell Line: Δ 2-7 DK DY5

In vitro

FIGURE 5

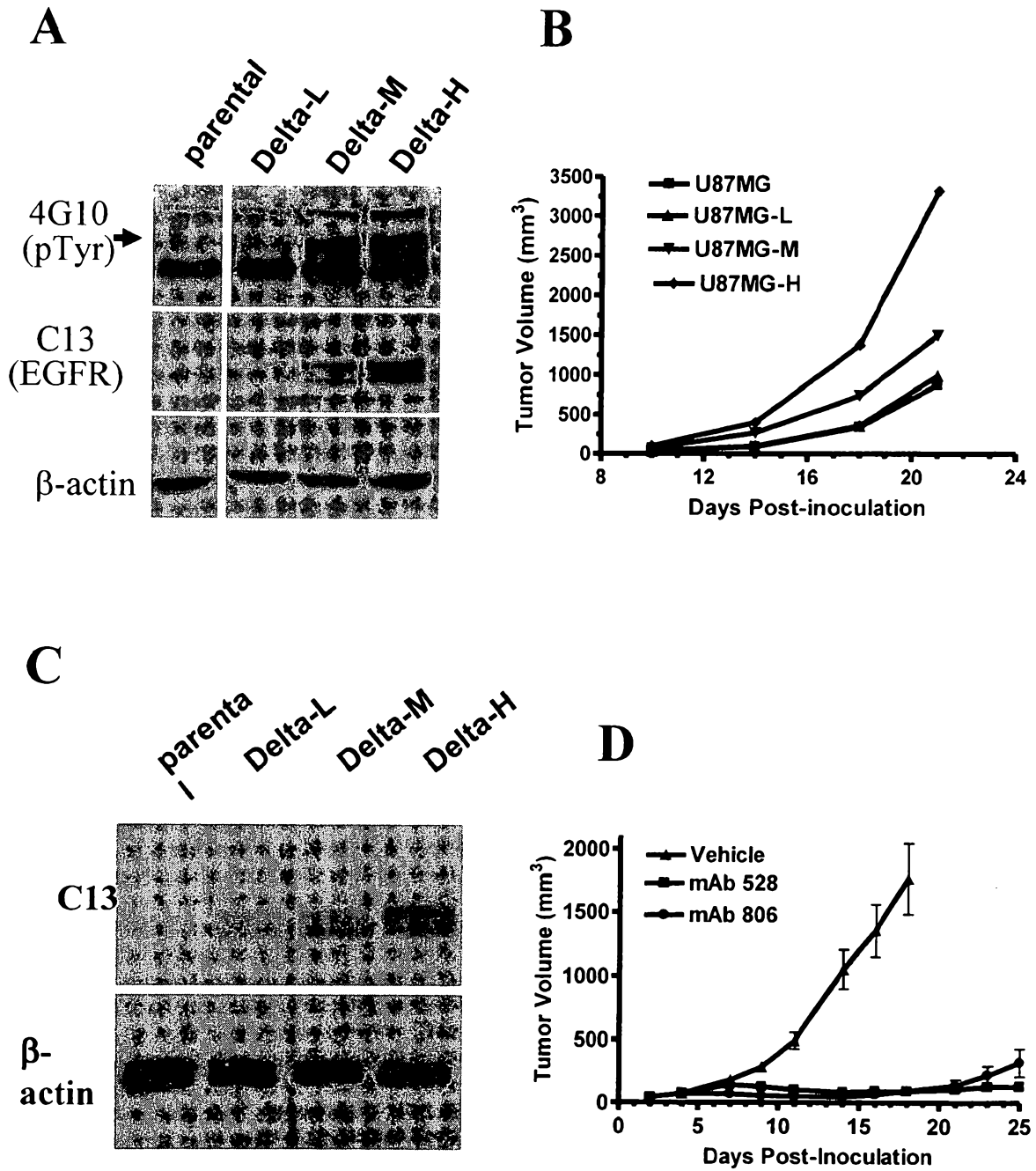


FIGURE 6

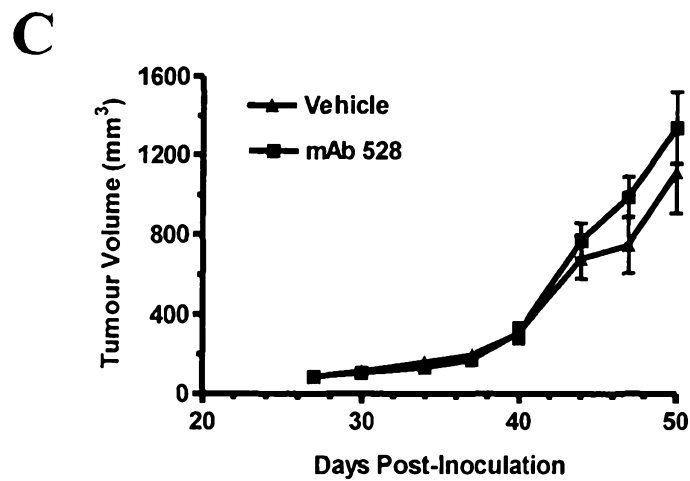
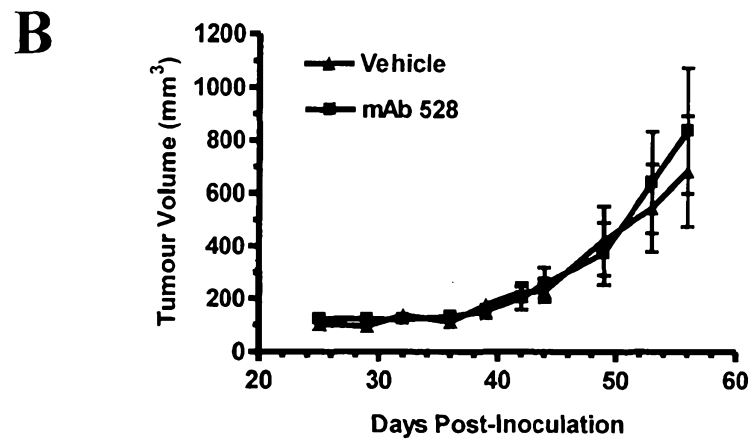
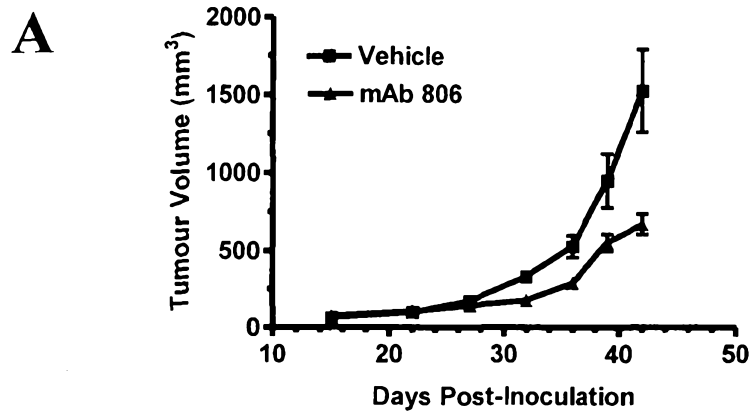
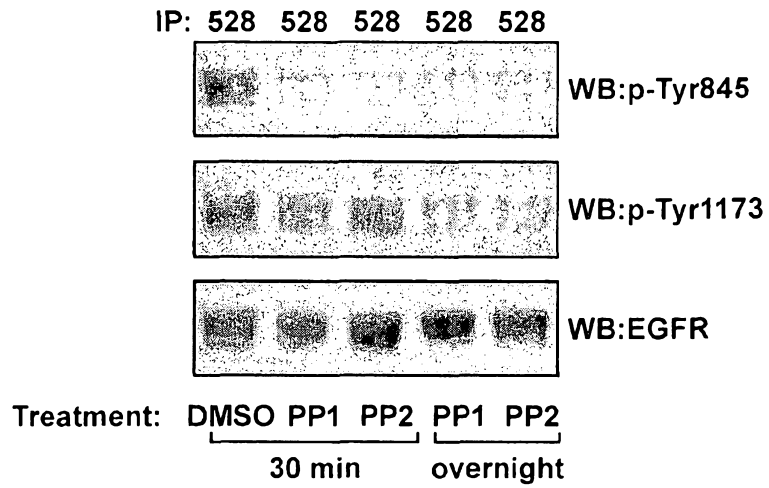
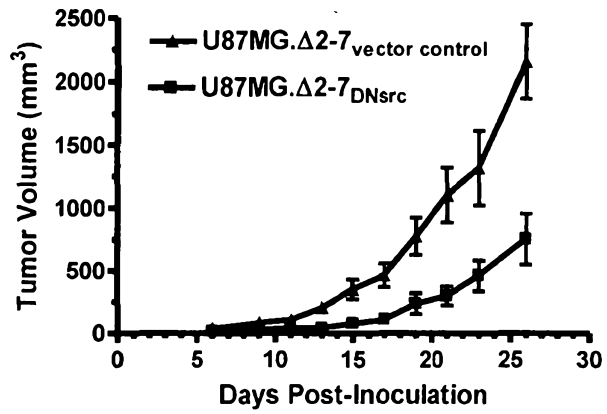


FIGURE 7

A



B



C

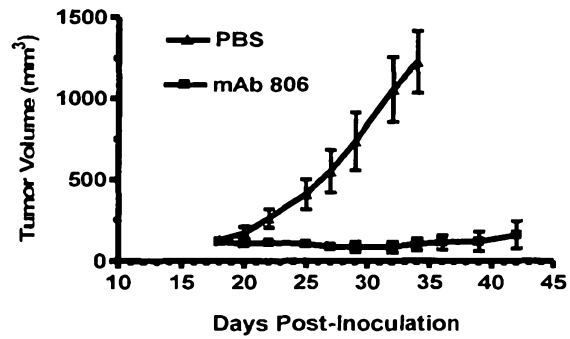


FIGURE 8

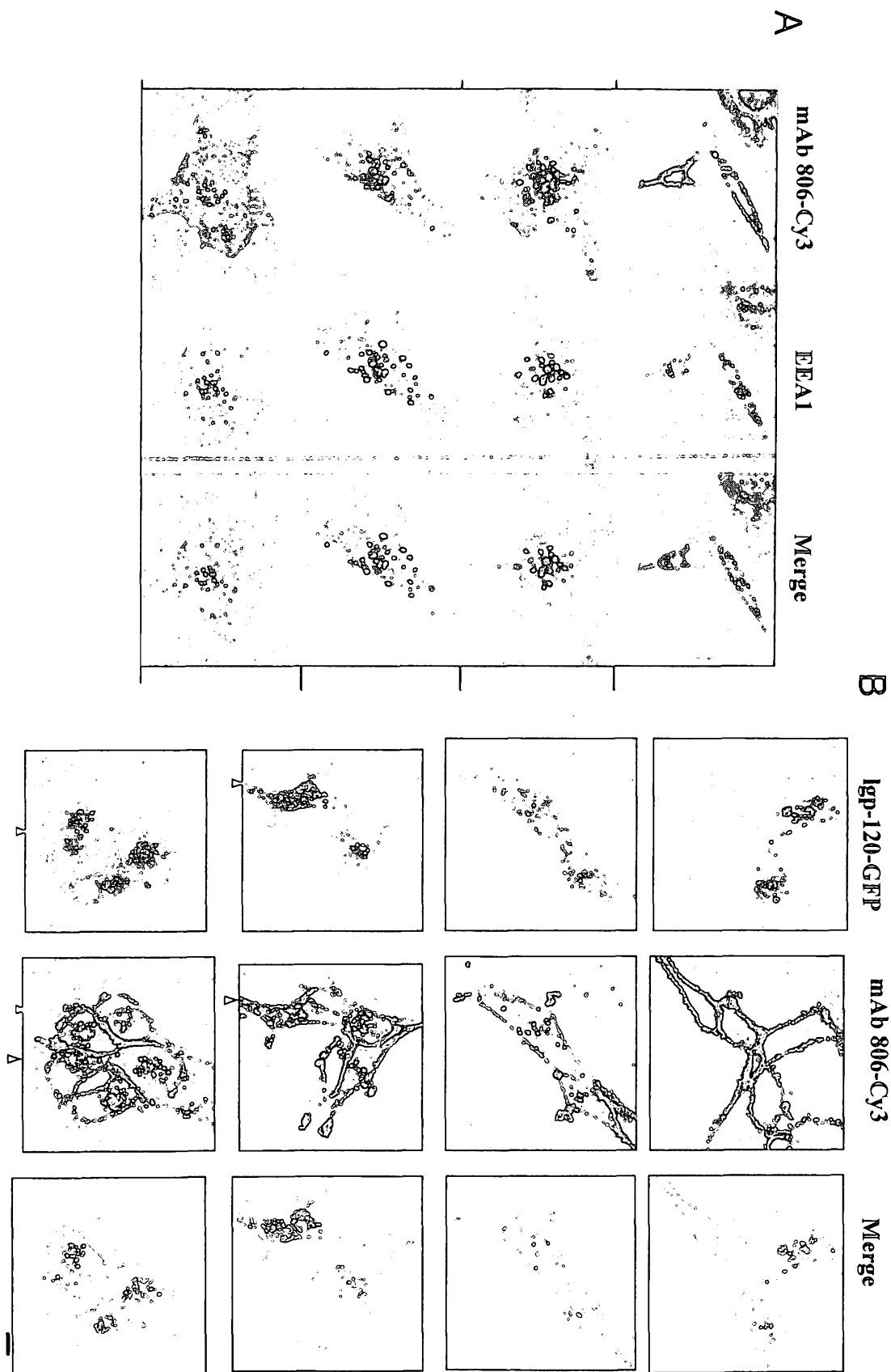


FIGURE 9

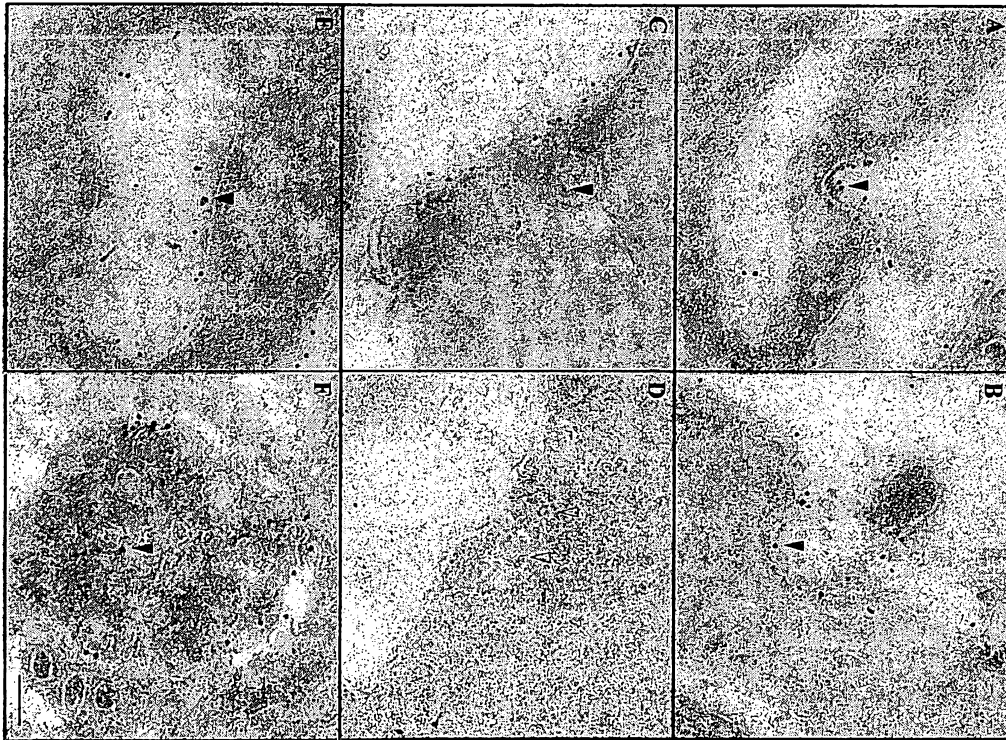
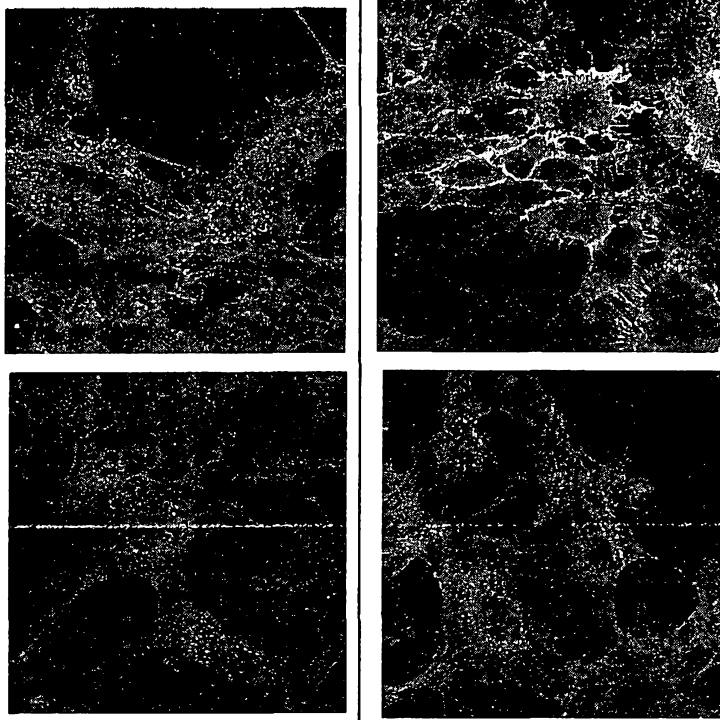
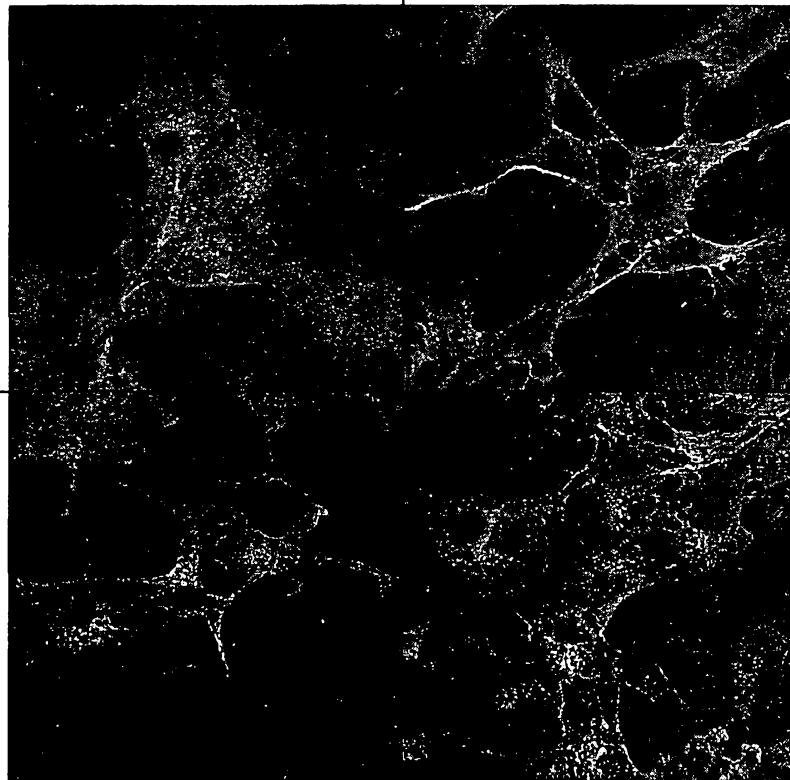


FIGURE 10



mAb 806



mAb 528

FIGURE 11

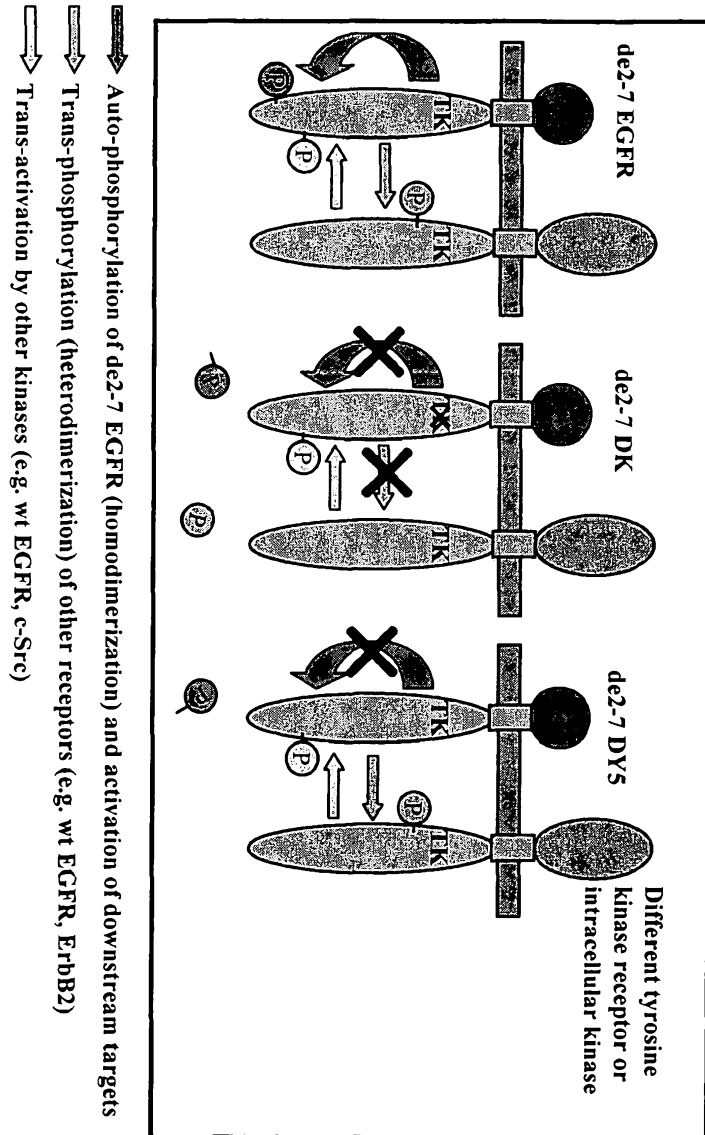


FIGURE 12

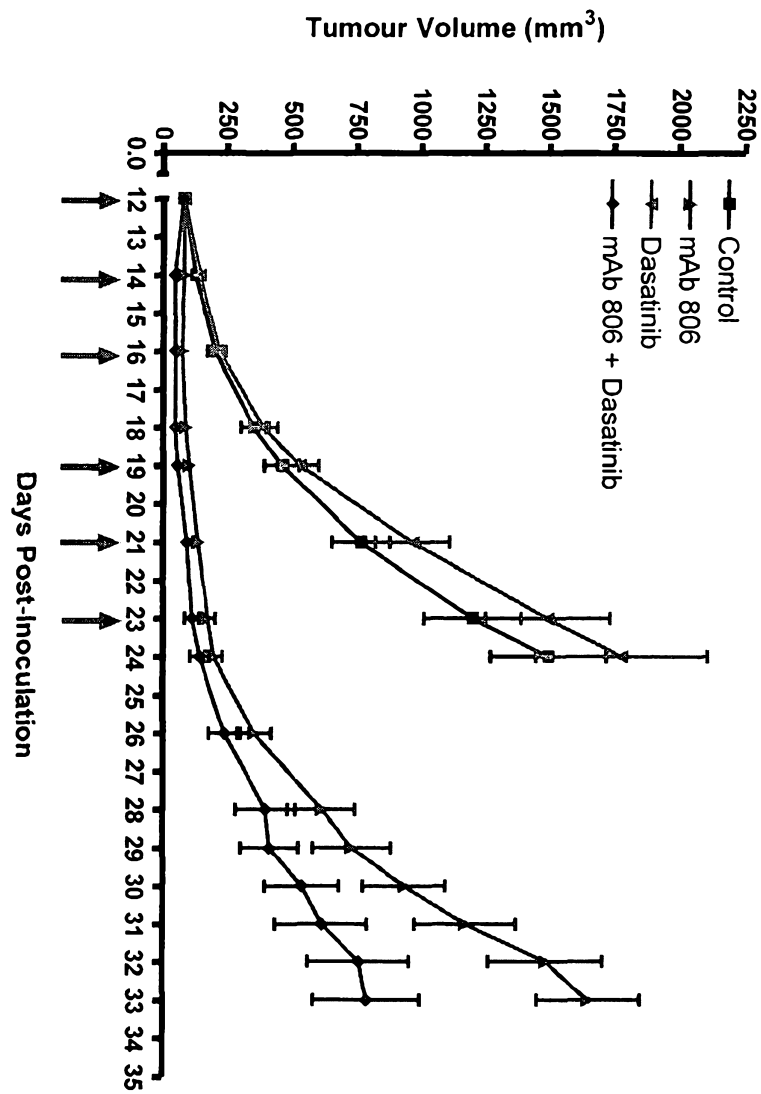


FIGURE 13

**Survival Curve Analysis
(Tumours $\geq 1500 \text{ mm}^3$):**

