EGFRvIII and c-Met pathway inhibitors synergize against PTEN-null/EGFRvIII+ glioblastoma xenografts

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Abstract
Receptor tyrosine kinase (RTK) systems, such as hepatocyte growth factor (HGF) and its receptor c-Met, and epidermal growth factor receptor (EGFR), are responsible for the malignant progression of multiple solid tumors. Recent research shows that these RTK systems comodulate overlapping and dynamically adaptable oncogenic downstream signaling pathways. This study investigates how EGFRvIII, a constitutively active EGFR deletion mutant, alters tumor growth and signaling responses to RTK inhibition in PTEN-null/HGF+/c-Met+ glioma xenografts. We show that a neutralizing anti-HGF monoclonal antibody (L2G7) potently inhibits tumor growth and the activation of Akt and mitogen-activated protein kinase (MAPK) in PTEN-null/HGF+/c-Met+/EGFRvIII−U87 glioma xenografts (U87wt). Isogenic EGFRvIII+ U87 xenografts (U87-EGFRvIII), which grew five times more rapidly than U87-wt xenografts, were unresponsive to EGFRvIII inhibition by erlotinib and were only minimally responsive to anti-HGF monoclonal antibodies. EGFRvIII expression diminished the magnitude of Akt inhibition and completely prevented MAPK inhibition by L2G7. Despite the lack of response to L2G7 or erlotinib as single agents, their combination synergized to produce substantial antitumor effects (inhibited tumor cell proliferation, enhanced apoptosis, arrested tumor growth, prolonged animal survival), against subcutaneous and orthotopic U87-EGFRvIII xenografts. The dramatic response to combining HGF:c-Met and EGFRvIII pathway inhibitors in U87-EGFRvIII xenografts occurred in the absence of Akt and MAPK inhibition. These findings show that combining c-Met and EGFRvIII pathway inhibitors can generate potent antitumor effects in PTEN-null tumors. They also provide insights into how EGFRvIII and c-Met may alter signaling networks and reveal the potential limitations of certain biochemical biomarkers to predict the efficacy of RTK inhibition in genetically diverse cancers. [Mol Cancer Ther 2009;8(7):1751–60]

Introduction
Understanding the molecular/genetic background of cancer has provided a foundation for targeted molecular therapies. Receptor tyrosine kinases (RTK) and their cognate ligands are key modulators of intracellular signaling and have emerged as potent regulators of molecular/cellular events involved in tumor malignancy. Common oncogenic changes in RTK/ligand systems include overexpression, gene amplification, activating mutations, and activating deletions. Examples include amplification of platelet-derived growth factor receptor (pdgfr) and epidermal growth factor receptor (egfr), overexpression of c-Met and/or its cognate ligand hepatocyte growth factor/scatter factor (HGF), activating c-met and egfr mutations, and the most common egfr gene rearrangement, EGFRvIII (an in-frame deletion of amino acids 6–273, resulting in a constitutively activated receptor; ref. 1). Coexpression of multiple RTK aberrations can activate overlapping and/or parallel oncogenic pathways in a multitude of genetically heterogeneous solid tumors (1). These parallel and overlapping pathways have the potential to limit the efficacy of single-agent–targeted therapeutics and offer potential mechanisms for drug resistance. This is exemplified by recent findings that c-Met pathway activation can provide a mechanism by which lung carcinomas escape EGFR inhibitors (2, 3). Recent in vitro experiments have revealed a phenomenon termed “RTK switching” whereby distinct RTKs act as independent but redundant inputs to maintain flux through downstream oncogenic signaling pathways when the seemingly dominant RTK is inhibited (4).

The HGF:c-Met pathway is overactivated by receptor/ligand overexpression and less commonly by activating receptor mutations or c-Met gene amplification in many solid tumors, including bladder, breast, colorectal, gastric, head and neck, kidney, liver, lung, pancreas, prostate, and thyroid carcinomas, gliomas, sarcomas, melanomas, and leukemias (5). HGF:c-Met pathway activation is associated with malignant progression and poor prognosis in many of these cancers (5). C-Met efficiently activates the phosphatidylinositol

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7 Also see http://www.vai.org/met.
Cooperation between HGF and EGFRvIII Inhibitors

inhibitors (17). All cells were grown at 37°C in a humidified incubator with 5% CO2.

Materials and Methods

Cell Culture and Reagents

U87MG cell lines were originally obtained from the American Type Culture Collection and grown in MEM with Earle Salts and l-glutamine (MEM 1×; Mediatech, Inc.) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Inc.), 2 mmol/L sodium pyruvate (Mediatech, Inc.), 0.1 mmol/L MEM-nonessential amino acids (Mediatech, Inc.), and penicillin-streptomycin (Mediatech, Inc.). U87-EGFRvIII cells were a kind gift from Dr. Gregory Riggins (15, 16), Johns Hopkins University School of Medicine, and were grown in DMEM high glucose with l-glutamine and sodium pyruvate (Mediatech, Inc.) supplemented with 10% fetal bovine serum, 1% of 10 mmol/L MEM-nonessential amino acids, and penicillin-streptomycin as previously described (17). All cells were grown at 37°C in a humidified incubator with 5% CO2.

Tumor Xenografts

Glioma xenografts were generated as previously described (17). Female 6- to 8-wk-old mice (National Cancer Institute) were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (5 mg/kg).

For s.c. xenografts, nu/nu mice received 4 × 10⁶ cells in 0.05 mL of PBS s.c. in the dorsal flank. When tumors reached ∼200 mm³, the mice were randomly divided into groups (n = 5 per group) and received the indicated doses of either L2G7 or isotype-matched control mAb (5G8) in 0.1 mL PBS i.p. as previously described (9). Tumor volumes were estimated by measuring two dimensions [length (a) and width (b)] and calculated using the equation: \( V = \frac{1}{2}a^2b \) (9, 18). At the end of each experiment, tumors were excised and frozen in liquid nitrogen, and protein was extracted for immunoblot analysis. Tumor doubling time (DT) was calculated using the formula: \( DT = \frac{\ln 2}{\ln 2/\ln (V_2 / V_1)} \) (ref. 19).

For intracranial xenografts, SCID/Beige mice received 1 × 10⁵ cells/2 μL by stereotactic injection into the right cuneate/putamen (17). L2G7 or 5G8 mAb was administered i.p. twice per week as described. Groups of mice (n = 5) were sacrificed by perfusion-fixation at the indicated times and the brains were removed for histologic studies. Tumor volumes were quantified by measuring the largest tumor cross-sectional area on H&E-stained cryostat sections using computer-assisted image analysis as previously described (17). Tumor volumes were estimated based on the formula: \( \text{vol} = (\text{square root of maximum cross-sectional area})^3 \) (ref. 20).

Antibodies

Antibodies were obtained from the following sources and used at the indicated dilutions: p-EGFR Tyr1068 (rabbit, 1:750), cleaved caspase-3 (rabbit, 1:150) and EGFR (rabbit, 1:1,000), phospho-Akt-Ser473 (rabbit, 1:1,000), phospho-p44/42 MAPK-Thr202/Tyr204 (rabbit, 1:1,000), phospho-Met-Tyr1234/1235 (rabbit, 1:500), and total-Met (mouse, 1:200) from Cell Signaling Technology; actin (rabbit, C-11; 1:1,000) from Santa Cruz Biotechnology; total Akt (mouse, 1:500), total MAPK (mouse, 1:1,000) from BD Sciences; secondary antibodies labeled with spectrally distinct near-IR dyes IRDye 800CW (goat anti-rabbit, 1:15,000) and IRDye 680CW (goat anti-rabbit, 1:20,000) from LI-COR Biosciences; anti-Ki67-K2 from Ventana Medical Systems; and anti-laminin (rabbit, 1:1,000) from Sigma.

L2G7 Iodination

Radioiodine [¹²⁵I]NaI, carrier-free, 2,125 Ci/mmol, was purchased from MP Biomedicals. Purified mAb was iodinated using the IODO-GEN method from Pierce according to the manufacturer’s instructions. Conjugated mAb was subjected to Sephadex G-25 desalting column chromatography (Amersham Biosciences) to remove unincorporated radioiodine. Radiochemical yields were typically 30% to 40%. Radiochemical purity met or exceeded 95% as determined by instant TLC. Specific radioactivities typically ranged from 150 to 180 μCi/μg. Antibodies were used for biodistribution studies within 1 h of radiolabeling. Retention of HGF binding activity following radioiodination was comparable with unlabeled L2G7 as determined by ELISA.

Biodistribution of L2G7

SCID mice bearing glioma xenografts received a single tail vein injection of ∼70 Bq of [¹²⁵I]L2G7. Three to four mice

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were sacrificed at each time point. Portions of ipsilateral brain hemispheres containing tumor xenograft, contralateral tumor-free hemisphere, and other organs were removed. The organs were weighed and the tissue radioactivity was measured with an automated gamma counter (1282 Compugamma CS; Pharmacia/LKB Nuclear, Inc.). The percent injected dose per gram of tissue (%ID/g) was calculated by comparison with samples of a standard dilution of the initial dose. All measurements were corrected for radioactive decay.

Single photon emission computerized tomography/computerized tomography (SPECT/CT) scanning was done essentially as previously reported with minor modifications (21). Mice received ~37 MBq of $^{125}$I-L2G7 in saline by single tail vein injection. Every 48 h, the mice were anesthetized using 3% isoflurane in oxygen and maintained using 1% isoflurane in oxygen. The mice were positioned on the X-SPECT (Gamma Medica) gantry and scanned using two low-energy, high-resolution pinhole collimators (Gamma Medica) rotating through 360° in 6° increments for 60 s per increment. Immediately following SPECT acquisition, we scanned the mice by CT (X-SPECT) over a 4.6-cm field-of-view using a 600-μA, 50-kV beam. The SPECT and CT data were coregistered using commercially available software (Gamma Medica) and displayed using AMIDE. 8

**Erlotinib Treatments**

Erlotinib tablets (Genentech, Inc.) were crushed and suspended in Ora-Plus Oral Suspending Vehicle (Paddock Laboratories, Inc.) and then diluted 4-fold in PBS. The animals received 0.2 mL of the erlotinib solution (150 mg/kg) by oral gavage 6 d/wk as previously described (22).

The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols used in this study.

**Immunohistochemistry**

Cryostat sections were stained with anti–cleaved caspase-3 and anti–MIB-1 antibodies as previously described (17). Biotinylated-conjugated secondary antibodies followed by incubation with 3,3′-diaminobenzidine peroxidase substrate was used to detect primary antibodies. Anti-MIB-1– and anti–cleaved caspase 3–stained sections were counterstained with Gill’s hematoxylin solution and methyl green, respectively. Proliferation and apoptotic indices were determined by computer-assisted quantification using ImageJ Software 9 essentially as previously reported (17).

**Immunoblot Analyses**

Total protein was extracted from glioma xenografts and from cells using radioimmunoprecipitation assay buffer (1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing fresh 1× protease and 1× phosphatase inhibitors (Calbiochem) at 4°C. Tissue extracts were sonicated on ice and centrifuged at 5,000 rpm at 4°C for 5 min. Supernatants were assayed for protein concentrations by Coomassie protein assay (Pierce) according to the manufacturer’s recommendations. Aliquots of 40 μg of total protein were combined with Laemmlı loading buffer containing β-mercaptoethanol and subjected to SDS-PAGE according to the method of Towbin et al. with some modifications (23, 24). For immunoblot analyses, proteins were electrophoretically transferred to nitrocellulose membranes with a semidy transfer apparatus (GE Healthcare) at 50 mA for 60 min. Membranes were subjected to quantitative dual-wavelength IR immunofluorescence using the methods described by Kearn et al. (25).10 This method allows the quantification of phosphorylated relative to the respective total protein species in the same samples by simultaneously staining blots with two secondary antibodies conjugated with spectrally distinct near-IR dyes. Membranes were incubated for 1 h in Odyssey Licor Blocking Buffer at room temperature and then overnight simultaneously with both relevant primary antibodies (anti-phospho and anti-total) at 4°C in 5% bovine serum albumin in TBS containing 0.1% Tween 20 (TBS/T). Membranes were then washed three times with TBS/T, incubated simultaneously with two secondary antibodies (IRDye 800CW goat anti-mouse 1:15,000, IRDye 680CW goat anti-rabbit 1:20,000; LI-COR Biosciences) for 1 h in TBS/T, washed three times with TBS/T, followed by washing twice with TBS. Proteins were detected and quantified using the Odyssey Infrared Imager (LI-COR Biosciences).

**Statistical Methods**

Statistical analysis consisted of one-way ANOVA followed by the Tukey or Dunnet’s multiple comparison test using Prism (GraphPad software Inc.). Survival data were analyzed with log analysis of survival curves using GraphPad Software. P values were determined for all analyses and P < 0.05 was considered significant. All experiments reported here represent at least three independent replications. Data are represented as mean values ± SE.

**Results**

**Biodistribution of $^{125}$I Anti-HGF L2G7 in Mice Bearing Intracranial Tumor Xenografts**

We showed previously that systemic L2G7 inhibits the growth and/or induces the regression of intracranial PTEN-null/HGF+/c-Met+ tumor xenografts (U87wt). However, the bioavailability of anti-HGF L2G7 to intracranial tumors had not been evaluated. We examined the delivery of radiolabeled L2G7 mAb to the brains of mice bearing pre-established intracranial U87wt glioma xenografts. Mice bearing U87wt xenografts in the right caudate/putamen received $^{125}$I-L2G7 by a single tail vein injection and delivery to the brain was quantified. $^{125}$I-L2G7 was preferentially localized to the tumor-bearing brain hemisphere as early as 12 hours and peaked 36 hours postinjection (Fig. 1A). The tumor-bearing brain hemisphere showed significantly higher radioactivity (10- to 20-fold higher) compared with

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8 http://amide.sourceforge.net/
9 http://rsb.info.nih.gov/ij/
10 Also see http://www.licor.com.
Results shown are representative of three replicate experiments.

Figure 1. Delivery of iodinated anti-HGF mAb L2G7 to orthotopic glioma xenografts. A, [125I]L2G7 was administered at time zero via tail vein to mice bearing right hemispheric U87wt glioma xenografts. Animals were sacrificed at the indicated postinjection times, and the percent-injected dose per gram (% ID/g) within tumor-bearing (ipsilateral) and contralateral brain hemispheres was determined. B, SPECT-CT done 48 h after a single i.v. injection of [125I]L2G7 shows localized increase of radioactivity within the right hemispheric tumor. n = 4; *, P < 0.01, compared with determination in ipsilateral brain at same time point. Results shown are representative of three replicate experiments.

We hypothesized that changes in Akt and/or MAPK activation (i.e., phosphorylation) would serve as biomarkers of HGF neutralization by anti-HGF mAbs. Mice bearing preestablished orthotopic U87wt tumor xenografts that were PTEN-null/HGF+/c-Met+/EGFRvIII+ were treated with three doses of either L2G7 or control 5G8 mAb every 2 days. Twenty-four hours after the last treatment, total tumor tissue protein was evaluated for phospho-Akt (Ser473) and phospho-p44/42 MAPK (Thr202/Tyr204) relative to total Akt and MAPK, respectively, by dual near IR immunoblot analysis (Fig. 3). Anti-HGF therapy significantly inhibited Akt and MAPK phosphorylation by ∼70% (P < 0.001) compared with control mAb that had no effect. These results show that Akt and MAPK pathways in orthotopic U87wt xenografts are inhibited by systemic anti-HGF therapy.

EGFRvIII Partially Abrogates the Effects of Anti-HGF Therapy on the Growth and Oncogenic Signaling of Subcutaneous Tumor Xenografts

We compared xenografts derived from U87wt cells with xenografts derived from U87wt cells engineered to express the constitutively active EGFR deletion mutant EGFRvIII (U87-EGFRvIII). Immunoblot analyses show that U87-EGFRvIII cells display hyperactivation of Akt and MAPK in comparison with U87wt cells (1.8-fold and 3-fold, respectively; Fig. 4A). Thus, transgenic EGFRvIII was functional and activated oncogenic pathways shared by both EGFR and c-Met. As expected, both the U87-EGFRvIII and the U87wt cells also express low levels of full-length wild-type HGF (see Supplementary Fig. S1).11

We compared the responses of preestablished subcutaneous U87wt and U87-EGFRvIII tumor xenografts to anti-HGF therapy. Tumor xenografts measured ∼250 mm3 before initiating treatment with either control mAb 5G8 or anti-HGF L2G7 (5 mg/kg, i.p. every 2 to 3 days). Untreated U87-EGFRvIII xenografts grew much more aggressively than untreated U87wt tumors with doubling times of 2.7 and 3.6 days, respectively (Fig. 4B and C; note different Y-axis scales). Anti-HGF L2G7 therapy markedly reduced growth of U87wt xenografts at a rate of 50% every 3.5 days (Fig. 4B). L2G7 therapy generated only modestly inhibited growth of U87-EGFRvIII xenografts as evidenced by a doubling time of 3.2 days (versus 2.7 days in controls) and tumors that were 40% smaller than controls (P = 0.05) at treatment day 8 (postimplantation day 18; Fig. 4C).

To test the hypothesis that tumor growth responses would translate to downstream cell signaling responses, the effects of anti-HGF therapy on Akt and MAPK activation was also examined in the two models. In U87wt

11 Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
xenografts, L2G7 significantly inhibited AKT phosphorylation by ∼70% to 80% (P < 0.05) and MAPK phosphorylation was inhibited by ∼50% to 60% (P = 0.05; Fig. 4D and F). Akt and MAPK inhibition developed ∼72 hours after initiating anti-HGF therapy and coincided with the timing of tumor regression. The magnitude of Akt inhibition by anti-HGF therapy was substantially less in U87-EGFRvIII xenografts (∼30%, P < 0.001) than in the U87wt tumors (compare Fig. 4D and E). EGFRvIII expression completely abrogated MAPK pathway inhibition by anti-HGF (compare Fig. 4F and G). These results suggested that the diminished sensitivity of U87-EGFRvIII xenografts to anti-HGF therapy is due, at least in part, to a shift from HGF:c-Met-dependent MAPK activation to HGF:c-Met-independent and presumably EGFRvIII-dependent MAPK pathway signaling.

HGF:c-Met and EGFR Pathway Inhibitors Synergistically Inhibit PTEN-Null/HGF+/c-Met+/EGFRvIII+ Xenografts

Our results suggested that simultaneously inhibiting the HGF:c-Met and EGFRvIII pathways would have additive or potentially cooperative antitumor effects on EGFRvIII+ glioma xenografts. Therefore, we examined the effects of anti-HGF L2G7 in combination with the EGFRvIII kinase inhibitor erlotinib on U87-EGFRvIII tumor growth and oncogenic cell signaling pathways. Animals bearing s.c. U87-EGFRvIII xenografts were treated with either L2G7 or control mAb 5G8 (5 mg/kg twice per week, i.p.), erlotinib (150 mg/kg 6 d/wk by oral gavage), or the combination of anti-HGF plus erlotinib. Anti-HGF therapy alone had no significant effect on U87-EGFRvIII tumor xenograft growth (Fig. 5A) although c-Met Tyr1244/1245 phosphorylation was

Figure 2. Systemic anti-HGF mAb inhibits growth of orthotopic PTEN-null/EGFRvIII+ glioma xenografts in a dose-dependent manner. Anti-HGF mAb L2G7 (0.625–10.0 mg/kg) or isotype control mAb 5G8 (10 mg/kg) was administered i.p. twice per week for five injections to mice bearing U87wt glioma xenografts. Treatment began 5 d after tumor cell implantation (postimplantation day (PID) 5). Animals were sacrificed on PID 23 and tumor volumes were quantified (A). H&E-stained brain sections show representative tumor xenografts (B). n = 5; *, P < 0.001 compared with control.
inhibited by ∼50% (Fig. 5C). Erlotinib alone had no effect on tumor growth (Fig. 5A) despite ∼85% inhibition of EGFRvIII Tyr 845 phosphorylation (Fig. 5B). Combining L2G7 and erlotinib markedly inhibited tumor growth and increased tumor doubling time from 2.9 to 7.7 days, seemingly through a synergistic mechanism (Fig. 5A). This response was consistent with a concomitant reduction in both c-Met and EGFRvIII phosphorylation (∼75% and 90% inhibition, respectively; Fig. 5B and C). Surprisingly, the robust inhibition of U87-EGFRvIII xenograft growth in response to L2G7 plus erlotinib occurred without reductions in either Akt or MAPK activation (i.e., phosphorylation; Fig. 5D and E).

We asked if the responses in the s.c. glioma xenografts described above would translate to orthotopic intracranial xenografts and to effects on animal survival. Preestablished orthotopic U87wt xenografts were treated with anti-HGF L2G7 or control mAb (i.p., 5 mg/kg) every 2 to 3 d for a total of three injections. Tumors were dissected from brain 24 h after the last dose and tumor cell lysates were subjected to quantitative dual-wavelength IR immunoblot analysis for the phosphorylated (P) and total (T) forms of Akt and MAPK as described in Materials and Methods. Representative bands of triplicate determinations are shown. n = 5; *, P < 0.05 compared with controls. Results shown are representative of three replicate experiments.

Figure 3. Systemic anti-HGF mAb therapy inhibits Akt and MAPK activation in orthotopic PTEN-null/EGFRvIII− xenografts. Mice bearing preestablished orthotopic U87wt xenografts were treated with anti-HGF L2G7 or control mAb (i.p., 5 mg/kg) every 2 to 3 d for a total of three injections. Tumors were dissected from brain 24 h after the last dose and tumor cell lysates were subjected to quantitative dual-wavelength IR immunoblot analysis for the phosphorylated (P) and total (T) forms of Akt and MAPK as described in Materials and Methods. Representative bands of triplicate determinations are shown. n = 5; *, P < 0.05 compared with controls. Results are representative of at least three replicate experiments.

Figure 4. EGFRvIII expression alters tumor xenograft growth and signaling responses to anti-HGF therapy. A, immunoblot analysis of whole cell protein isolated from U87wt and U87-EGFRvIII cell lines (cultured for 24 h in 0.1% serum conditions) shows increased levels of Akt and MAPK activation (phosphorylation) in cells expressing EGFRvIII. B and C, growth responses of s.c. U87wt and U87-EGFRvIII tumor xenografts (n = 5) to control mAb 5G8 or anti-HGF mAb L2G7 (5 mg/kg i.p., every alternate day). Arrows, days of each mAb injection. U87wt and U87-EGFRvIII tumors were of comparable size at the time of treatment initiation. Tumor lysates were obtained from U87wt (D and F) and U87-EGFRvIII (E and G) xenografts 24 h following each of the three L2G7 injections and subjected to quantitative dual-wavelength IR immunoblot analysis for the phosphorylated (P/T) and total (T) forms of Akt and MAPK as described in Materials and Methods. Levels of phospho/total Akt and MAPK are relative to control conditions that are normalized to 1.0. Representative bands of triplicate determinations are shown. *, P < 0.05; **, P < 0.001 compared with controls. Results are representative of at least three replicate experiments.
same on these postimplantation days. Erlotinib alone and L2G7 alone both reduced the size of U87-EGFRvIII tumors by ~50% compared with controls. Erlotinib plus L2G7 reduced the size of U87-EGFRvIII tumors ~6-fold compared with each monotherapy and ~15-fold compared with controls. Erlotinib alone had no effect on the growth of U87wt tumors that lack EGFR pathway hyperactivation. Furthermore, L2G7 alone and L2G7 plus erlotinib generated similar responses in U87wt xenografts. Thus, erlotinib (either alone or combined with anti-HGF) had no discernable effect in the EGFRvIII− U87wt xenografts.

The cooperative effects of L2G7 and erlotinib translated to a substantial improvement in the survival of animals bearing orthotopic EGFRvIII+ xenografts (Fig. 6B). Animals bearing preestablished intracranial U87-EGFRvIII glioma xenografts were treated with either control 5G8 or anti-HGF L2G7 mAb (5 mg/kg i.p. twice per week) with or without erlotinib (150 mg/kg 6 d/wk) from postimplantation days 5 to 21. Compared with controls, L2G7 alone and erlotinib alone had essentially no effect on median survival. All animals treated with either erlotinib or L2G7 were dead by postimplantation day 21. In contrast, all animals treated with erlotinib plus L2G7 survived beyond postimplantation day 21, the last day of therapy, and deaths in this treatment group only occurred after therapy was discontinued. Erlotinib plus L2G7 also extended median survival to 28 days, with 25% of animals surviving at 30 days, 9 days after stopping all therapy.

The cooperative/synergistic antitumor effects of L2G7 plus erlotinib in EGFRvIII+ xenografts can be explained, at least in part, by changes in tumor cell proliferation and apoptosis (Fig. 6C and D). Neither erlotinib nor L2G7 monotherapies affected tumor Ki-67 labeling (identifies cells within the cell cycle) or labeling with anti–cleaved caspase-3 (apoptosis marker) in U87-EGFRvIII+ xenografts. In contrast, erlotinib plus L2G7 reduced Ki67 labeling by ~25% and increased labeling with anti–cleaved caspase-3 ~6-fold (P < 0.05). The increase in U87-EGFRvIII apoptosis in response to erlotinib plus L2G7 was about twice that induced in U87wt tumors by either L2G7 or erlotinib monotherapy or their combination (Fig. 6D).

**Discussion**

Receptor tyrosine kinase inhibitors offer promising new treatments for solid malignancies. We and others have reported that HGF/c-Met pathway inhibitors can have potent antitumor effects in HGF+/c-Met+ preclinical tumor models (9–12). However, more information on how the genetic background and overlapping signaling networks influence tumor growth is needed to reap the full potential of these new agents. The constitutively active EGFR deletion mutant EGFRvIII is common in glioblastoma and can confer tumor cell resistance to tyrosine kinase inhibitors (26). This prompted us to investigate the influence of EGFRvIII on the tumor response to anti-HGF therapeutics. We compared the antitumor effects of a neutralizing anti-HGF mAb on isogenic EGFRvIII− and EGFRvIII+ U87 tumor xenografts that share a PTEN-null/HGF+/c-Met+ background. Our finding that EGFRvIII expression dramatically diminishes antitumor responses to HGF/c-Met+ preclinical tumor models (9–12). However, more information on how the genetic background and overlapping signaling networks influence tumor growth is needed to reap the full potential of these new agents. The constitutively active EGFR deletion mutant EGFRvIII is common in glioblastoma and can confer tumor cell resistance to tyrosine kinase inhibitors (26). This prompted us to investigate the influence of EGFRvIII on the tumor response to anti-HGF therapeutics. We compared the antitumor effects of a neutralizing anti-HGF mAb on isogenic EGFRvIII− and EGFRvIII+ U87 tumor xenografts that share a PTEN-null/HGF+/c-Met+ background. Our finding that EGFRvIII expression dramatically diminishes antitumor responses to HGF/c-Met+ pathway inhibition was not unexpected because EGFRvIII can activate shared downstream oncogenic signaling pathways independent of c-Met. However, the dramatic supra-additive and synergestic effects of combining EGFR and c-Met pathway inhibitors in EGFRvIII+ tumors that were essentially insensitive to...
either EGFR or c-Met inhibitors used individually was unexpected. These results are particularly relevant within the context of the relative high frequency of c-Met and EGFR coexpression/hyperactivation in many solid tumors. Recent clinical observations in lung carcinomas showing that c-Met amplification and activation can function as an escape mechanism for erlotinib-responsive cancers (2, 3) further support a treatment strategy combining c-Met and EGFR pathway inhibitors for gliomas that contain EGFRvIII and possibly hyperactivated EGFR.

Current paradigms for targeted therapeutics imply that inhibiting upstream kinases, such as c-Met and EGFR, will be ineffective in the presence of mutations in downstream signaling checkpoints, such as the tumor suppressor PTEN. Clinical observations in patients with genetically heterogeneous brain tumors have led to the conclusion that PTEN loss renders gliomas unresponsive to EGFR inhibitors presumably due to the diminished dependence of Akt activity on PI3K (27). Our finding that PTEN-null U87-EGFRvIII xenografts were insensitive to erlotinib monotherapy is consistent with these clinical observations. However, our results also show that PTEN loss does not necessarily render Akt activity or tumor growth insensitive to upstream receptor tyrosine kinases (i.e., c-Met, EGFRvIII) or their inhibition.

These results support the in vitro findings of Stommel et al., who conclude that when considering the targeting of multi-input signaling systems, predictors of clinical efficacy should be based on the total signal flux contributed by multiple pathways and signaling checkpoints (4). Furthermore, the unexpected synergism between anti-HGF mAb and erlotinib against the EGFRvIII+ glioma xenografts show that tumor subsets can have complex nonlinear codependencies on multiple receptor tyrosine kinases and presumably other molecular regulators of tumor growth and malignant progression (28). A possible example of this is described by Bonine-Summers et al. who showed that EGFR inhibition can block HGF activation of c-Met, an effect not attributable to a direct inhibition of Met by EGFR inhibitors (29).

Understanding each tumor’s signaling network and the molecular consequences of targeted inhibition should make it possible to combine targeted molecular therapeutics rationally. We initially predicted that two prominent downstream constituents of receptor tyrosine kinase pathways, Akt and MAPK, would serve as biochemical markers of c-Met and EGFRvIII pathway inhibition and tumor growth response. We observed EGFRvIII+ glioma regression concurrently with a substantial decline in both phospho-Akt and phospho-MAPK in response to anti-HGF monotherapy.
In contrast, anti-HGF failed to induce tumor regression or diminish phospho-MAPK in EGFRVIII xenografts. Surprisingly, adding erlotinib to anti-HGF therapy failed to reduce Akt and MAPK activation in EGFRVIII xenografts below levels seen in response to anti-HGF alone. These in vivo findings differ from the in vitro findings of Stommel et al. using cultured cell lines (4). Potential explanations include differences in the kinetics and magnitudes of c-Met and EGFRVIII inhibition achieved in vitro versus in vivo. For example, our tissue sampling times might not have been optimal for detecting dynamic and transient pathway inhibition in the EGFRVIII xenografts. Alternatively, there may be in vivo specific mechanisms of signaling network regulation such as stromal effects or compensatory secondary signaling responses that are not present in vitro. A careful histologic review of the xenografts revealed no differences in xenograft infiltration by nonneoplastic stromal components between the control EGFRVIII xenografts and those treated with erlotinib plus L2G7. An alternative possibility is that phospho-Akt and phospho-MAPK might not adequately reflect the flux through all relevant oncogetic signals driven by the combination of EGFRVIII and c-Met signaling, thereby implicating other critical downstream mediators. Recent reports have identified over 69 proteins within the signaling networks shared by the EGFR and Met pathways (28, 30). Considerable work is needed to identify their interactive roles and clinical utility. Our finding that erlotinib had minimal or no effects on Akt and MAPK phosphorylation even under conditions that reduced EGFRVIII Tyr845 phosphorylation by >80% may seem contradictory. However, this can be explained by the fact that the levels of phospho-EGFRVIII in erlotinib-treated tumors remained detectable and presumably above a critical threshold for Akt and MAPK activation (28). It is interesting that anti-HGF monotherapy reduced EGFRVIII Tyr845 phosphorylation by ~50%, suggesting receptor cross-talk either by direct receptor interactions or via indirect mechanisms such as src-dependent EGFR phosphorylation as observed by Tice et al. in wild-type EGFR (29, 31).

The marginal clinical efficacy observed to date for RTK inhibitor monotherapy in malignant brain tumors that commonly coexpress activated c-Met and EGFRVIII is partially explained by our current findings within the context of previous results (4). We now provide in vitro evidence that inhibiting the contributions of multiple RTKs can be profoundly more beneficial than monotherapy directed at a single RTK. This evidence further supports the application of a “personalized medicine” paradigm, combining EGFR and c-Met pathway inhibitors in patients with gliomas containing the appropriate molecular profiles (4, 32, 33). It will be important to determine if our results can be extended to other RTKs that are amplified or mutated, and applied to improving clinical outcomes. The recent entry of novel c-Met pathway inhibitors into phase I/II clinical trials, and the availability of Food and Drug Administration–approved EGFR inhibitors, establish a therapeutic armamentarium to test these findings in the clinical setting (34–38).

Disclosure of Potential Conflicts of Interest

J. Kim: employee with ownership interest, Galaxy Biotech, Inc. B. Lal and J. Laterra: licensing agreement with Galaxy Biotech, Inc. No other potential conflicts of interest were disclosed.

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EGFRvIII and c-Met pathway inhibitors synergize against PTEN-null/EGFRvIII + glioblastoma xenografts


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