Hepatocyte Growth Factor Sensitizes Brain Tumors to c-MET Kinase Inhibition

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Abstract

Purpose: The receptor tyrosine kinase (RTK) c-MET and its ligand hepatocyte growth factor (HGF) are deregulated and promote malignancy in cancer and brain tumors. Consequently, clinically applicable c-MET inhibitors have been developed. The purpose of this study was to investigate the not-well-known molecular determinants that predict responsiveness to c-MET inhibitors and to explore new strategies for improving inhibitor efficacy in brain tumors.

Experimental Design: We investigated the molecular factors and pathway activation signatures that determine sensitivity to c-MET inhibitors in a panel of glioblastoma and medulloblastoma cells, glioblastoma stem cells, and established cell line–derived xenografts using functional assays, reverse protein microarrays, and in vivo tumor volume measurements, but validation with animal survival analyses remains to be done. We also explored new approaches for improving the efficacy of the inhibitors in vitro and in vivo.

Results: We found that HGF coexpression is a key predictor of response to c-MET inhibition among the examined factors and identified an ERK/JAK/p53 pathway activation signature that differentiates c-MET inhibition in responsive and nonresponsive cells. Surprisingly, we also found that short pretreatment of cells and tumors with exogenous HGF moderately but statistically significantly enhanced the antitumor effects of c-MET inhibition. We observed a similar ligand-induced sensitization effect to an EGF receptor small-molecule kinase inhibitor.

Conclusions: These findings allow the identification of a subset of patients that will be responsive to c-MET inhibition and propose ligand pretreatment as a potential new strategy for improving the anticancer efficacy of RTK inhibitors.

Introduction

The receptor tyrosine kinase (RTK) c-MET and its ligand hepatocyte growth factor (HGF) are key determinants of malignancy in human cancers including brain tumors. c-MET is aberrantly activated in high-grade gliomas and embryonal brain tumors and activation is associated with poor clinical outcomes (1, 2). c-MET activation enhances malignancy by inducing cell proliferation, survival, migration, invasion, promoting tumor angiogenesis, and supporting a stem cell phenotype (3–7). Inhibiting endogenous c-MET and/or HGF in experimental tumors leads to growth inhibition and tumor regression (8–14). The oncogenic effects of c-MET are mediated by signaling networks that include the Ras/MAPK and PI3K/Akt pathways (15–18). c-MET pathway activation is regulated by several factors including ligand and receptor expressions, cross-talk with the EGF receptor (EGFR; refs. 19–22), and modulation of c-MET–dependent signaling by the tumor suppressor PTEN (23, 24).

Clinically translatable c-MET inhibitors have been developed (13, 25–27). Prominent among these are small molecules that target the catalytic activity of the kinase. Four selective kinase inhibitors (PF-02341066, ARQ197, JNJ-38877605, PF-04217903) and 4 broad-spectrum kinase inhibitors (XL184, PM470, MGCD265, MK-2461) have entered initial clinical evaluations (28). PF-02341066, also known as crizotinib (METi; Pfizer), is a clinically applicable, potent and selective ATP competitive small-molecule kinase inhibitor of c-MET (13, 29). The sensitivity of cancer cells to c-MET inhibition varies, and the factors that determine this sensitivity have not been systematically studied to date (30). However, the efficient use of c-MET inhibitors requires an understanding of these factors to identify responsive patients.
In this study, we used a panel of brain tumor cell lines, primary cells, glioblastoma stem cells, and xenografts to investigate the factors that determine sensitivity to c-MET inhibition. We found that HGF coexpression is a key predictor of sensitivity to METi among the tested factors and identified an ERK/JAK/p53 pathway activation signature that differentiates responsive from nonresponsive tumor cells. Furthermore, we discovered for the first time that short-term exogenous HGF treatment of tumor cells and xenografts sensitizes them to METi. Similarly, short-term exogenous EGF treatment sensitized tumor cells to the antitumor effects of the EGFR kinase inhibitor erlotinib. These findings have important practical implications for the clinical use of recently developed c-MET (and possibly also other RTK) inhibitors in cancer and brain tumors. They identify a subset of patients that are more likely to respond to c-MET inhibition and that therefore could be preselected for such therapies. They also uncover ligand pretreatment as a potential new strategy for improving the efficacy of RTK inhibitors.

**Translational Relevance**

Our study identifies hepatocyte growth factor (HGF) coexpression as one key predictor of brain tumor sensitivity to a clinically applicable small-molecule kinase inhibitor (METi) of the receptor tyrosine kinase (RTK) c-MET and uncovers an ERK/JAK/p53 pathway activation signature that differentiates responsive from nonresponsive tumor cells. The study also shows for the first time that short-term exogenous HGF treatment of tumor cells and xenografts sensitizes them to METi. Similarly, short-term exogenous EGF treatment sensitized tumor cells to the antitumor effects of the EGFR kinase inhibitor erlotinib. These findings have important practical implications for the clinical use of recently developed c-MET (and possibly also other RTK) inhibitors in cancer and brain tumors. They identify a subset of patients that are more likely to respond to c-MET inhibition and that therefore could be preselected for such therapies. They also uncover ligand pretreatment as a potential new strategy for improving the efficacy of RTK inhibitors.

**Materials and Methods**

**Reagents and cells**

The c-MET kinase inhibitor METi (PF-2341066) [(R)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine] was from Pfizer. The EGFR inhibitor, erlotinib, was from Sigma. Human glioblastoma cell lines (U87, A172, U373, T98G, U1242, SF-767), primary cells (GBM-6, GBM-10), glioblastoma stem cells (GSC; 1228, 0308), medulloblastoma cell lines (DAOY, PFSK, D425, ONS-76), and xenografts were used in this study. U87, A172, U373, T98G, and DAOY were from American Type Culture Collection. U1242 and SF-767 were kind gifts from Dr. Isa Hussaini (University of Virginia, Charlottesville, VA), Dr. Jasti Rao (University of Illinois, Chicago, IL), and Dr. Russel Pieper (UCSF, San Francisco, CA), respectively. Primary glioblastoma cells (GBM-6 and GMB-10 at 5–10 passages), a gift from Dr. Jann Sarkaria (Mayo Clinic, Rochester, MN), were isolated from patients who underwent surgery at the Mayo clinic and were molecularly and functionally characterized (31, 32). GSCs 1228 and 0308 were a gift from Dr. Howard Fine (NIH, Baltimore, MD; ref. 33). PFSK, D425, and ONS-76 cells were a gift from Dr. Charles Eberhart (Johns Hopkins University, Baltimore, MD). The cells were cultured as described in the Supplementary Methods.

**Treatment with METi and erlotinib**

The cells were grown overnight in low-serum media and treated with METi (30–300 nmol/L) or erlotinib (2 μmol/L) for 1 hour before treatment with or without 20 ng/mL HGF or 100 ng/mL EGF, respectively. For *in vivo* sensitivity studies, mice with established intracranial glioblastoma xenografts were randomly separated into control and experimental groups. Treatment groups consisted of vehicle water or 25 mg/kg METi administered by daily oral gavage from days 7 to 28 posttumor implantation. For METi *in vivo* sensitization studies, subcutaneous GSC xenografts were injected with either PBS or HGF (400 ng/cm²) for 2 hours before treatment with METi (20 μL/cm³ tumor volume). The animals were treated for 2 weeks before tumor removal and measurement of tumor weight.

**Immunoblotting**

Quantitative immunoblotting was conducted to measure protein expression and phosphorylation as previously described (3). Antibodies were used that are specific for c-MET, phospho-MET (p-MET), HGF, EGFR, and p-EGFR (Santa Cruz Biotechnologies), and PTEN (Cell Signaling Technology). All blots were stripped and reprobed with β-actin antibody (Santa Cruz Biotechnologies) as loading control. All antibodies were used at a 1:1,000 dilution. Quantitative analysis was conducted by densitometry on film (BioRad GS 800) and normalized to the β-actin measured on the same blots. Two independent immunoblotting experiments were carried out for all cell lines and quantified protein levels were averaged.

**Cell death and cell proliferation assays**

Cell death and apoptosis were assessed by Annexin-V-PE/7AAD flow cytometry as previously described (3). Cell samples were analyzed on a FACScan, and dead cell and apoptotic fractions were determined. Cell proliferation was assessed by cell counting for 5 days as previously described (3). All experiments were carried out 3 times using triplicate samples.

**Reverse-phase protein microarray construction and analysis**

Pathway activation mapping was conducted by reverse-phase protein microarray as previously described (34–36). The cells were treated with METi (300 nmol/L) or vehicle control in triplicates for 24 hours and lysed (more details in Supplementary Methods). Protein signaling analytes were chosen for analysis based on their previously described involvement in key aspects of c-MET-associated tumor
biology. Detection was conducted using a fluorescence-based signal amplification strategy using Streptavidin-conjugated IRDye680 (LI-COR Biosciences) as detection reagent. All antibodies were validated for single-band specificity and for ligand induction (for phospho-specific antibodies) by immunoblotting before use on the arrays as previously described (34–36). Each array was scanned using a TECAN LS (Vidar Systems Corporation). After scanning, spot intensity was analyzed, data were normalized to total protein, and a standardized, single data value was generated for each sample on the array by MicroVigene software V2.999 (VigenTe'ch).

**In vivo experiments**

*In vivo* sensitivity of glioblastoma cells to METi was assessed using an orthotopic xenograft mouse model. U87 (3 × 10⁶) and T98G (6 × 10⁵) were stereotactically implanted into the right striatum of severe combined immunodeficient (SCID) mice (n = 6 per treatment group). One week after implantation, the animals were treated with oral gavage of METi or control every day for 3 weeks at 25 mg/kg body weight. Four weeks after tumor implantation, the maximal tumor cross-sectional area was determined by computer-assisted image analysis of hematoxylin and eosin (H&E)-stained brain cross-sections, and the tumor volume was calculated using the formula: volume = (square root of maximal tumor cross-sectional area)³ as shown previously (37).

Flank xenografts were used to determine the effects bolus HGF injections on responses to METi. GSCs 1228 (3 × 10⁵) in 50% Matrigel were injected into the flanks of SCID mice (n = 6 per treatment group). When the tumors reached about 5 mm diameter, they were treated with intratumoral injections of HGF (400 ng/cm³ tumor volume) or control for 2 hours before treatment with METi (5 nmol/L at 20 µL/cm³ tumor volume). The animals were treated for 2 weeks. Tumor diameters were measured every other day, and tumors were weighted at the end of the experiment.

**Statistical analyses**

The statistical association between apoptosis (or proliferation) and protein expression was evaluated both with regression and Spearman (rank-based) correlation analyses. The latter analysis was conducted to robustly evaluate statistical significance of such association, even though the protein expression values were normally distributed for the regression analysis. To evaluate the statistical significance of the difference between each treated and control animal groups *in vivo*, we used both 2-sample *t* test and nonparametric Wilcoxon rank-sum test.

The continuous variable reverse-phase protein microrray data generated were subjected to both unsupervised and supervised statistical analyses. Statistical analyses were conducted on final microarray intensity values obtained using R version 2.9.2 software (The R Foundation for Statistical Computing). Quartile determination was conducted to determine which cell lines and treatment groups fell into the highest (75th percentile) and lowest (25th percentile) of HGF expression. If the distribution of variables for the analyzed groups were normal, a 2-sample *t* test was conducted. If the variances of 2 groups were equal, 2-sample *t* test with a pooled variance procedure was used to compare the means of intensity between 2 groups. Otherwise, 2-sample *t* test without a pooled variance procedure was adopted. For nonnormally distributed variables, the Wilcoxon rank-sum test was used. Significance levels were set at *P* < 0.05.

**Results**

**c-MET kinase inhibitor METi completely inhibits c-MET activation in all brain tumor cells**

We tested the efficacy of the c-MET kinase inhibitor METi in inhibiting c-MET activation to ensure that differences in antitumor response are not caused by differences in c-MET inhibition levels. We treated 14 different brain tumor cells with METi and measured c-MET phosphorylation relative to total c-MET protein using quantitative immunoblotting. METi was applied at different concentrations (30–300 nmol/L) for 1 hour before treatment of the cells with (or without) HGF (20 ng/mL) for 5 minutes. The cells included glioblastoma cells (U87, A172, T98G, U373, SF-767, and U1242), GSCs (O308 and 1228), glioblastoma primary cells (GBM-6 and GBM-10), and medulloblastoma cells (DAOY, ONS-76, D425, and PF5). METi completely or almost completely inhibited both basal and HGF-induced c-MET phosphorylation at 300 nmol/L in all tested cells (Fig. 1A). We therefore used this concentration in all subsequent experiments.

The expressions of c-MET, p-MET, HGF, EGFR, p-EGFR, and PTEN vary in human glioblastoma and medulloblastoma cells

On the basis of published literature on c-MET functions, regulation, signaling, and interactions, we focused on c-MET, p-MET, HGF, PTEN, EGFR, and p-EGFR levels as the most likely predictors of antitumor responses to c-MET inhibition. We first used quantitative immunoblotting to measure the levels of the above proteins in all cells. Expression levels of the proteins varied significantly between the different cells (Fig. 1B and C). We also analyzed all cells for c-MET gene amplification, another potential predictor of responsiveness to c-MET inhibitors. No gene amplification was detected in any of the cells (data not shown). Overall, the cells exhibited significant variation in the factors that are most likely to predict responsiveness to c-MET inhibitors.

The effects of METi on death and apoptosis vary between the different brain tumor cells

To investigate the effects of METi on brain tumor cell death and apoptosis, cells were treated with METi (300 nmol/L) for 48 hours and assessed for cell death and apoptosis by Annexin-V/7AAD flow cytometry (n = 3). Total cell death and apoptosis levels induced by METi varied between the different cells. METi induced strong apoptosis in some cells such as U87, GBM-6, and D425 but had no
effect on other cells such as ONS-76 (Fig. 2A). These results indicate that METi induces brain tumor cell death at various degrees in the different cells.

The effects of METi on proliferation vary between the different brain tumor cells

To investigate the effects of METi on brain tumor cell proliferation, cells were incubated with METi (300 nmol/L) for 5 days in 0.01% FBS and counted every day. The experiments were repeated 3 times and growth curves were established. The inhibitory effect of METi on proliferation varied between the different tumor cell lines. METi strongly inhibited the proliferation of some cells such as U87, GBM-6, and D425 but had modest or no effects on other cells such as ONS-76 (Fig. 2B). Overall, the differential effects of METi on tumor cell proliferation were stronger than those on cell death but similar in their differential effects between the different cell lines. These results indicate that METi exerts
Figure 2. The effects of METi on cell death/apoptosis and proliferation vary between the different brain tumor cells. A, the glioblastoma cell lines U87, A172, T98G, U373, SF-767 and U1242; primary cells GBM-10 and GBM-6, GSCs 0308 and 1228; and the medulloblastoma cell lines D425, ONS-76, DAOY, and PFSK were treated with METi or control. The cells were subsequently assessed for apoptosis and cell death by Annexin-V/7AAD flow cytometry, and the percentage of dead cells was determined. B, the same brain tumor cells as in A were treated with METi or control. The cells were subsequently assessed for proliferation by cell counting over a period of 5 days and growth curves were established. All experiments were carried out in triplicates and repeated 3 times.
differential inhibitory effects on cell proliferation in different cells.

**HGF coexpression determines sensitivity to c-MET kinase inhibition**

To identify the factor(s) that determine responsiveness of brain tumor cells to METi-induced cell death and inhibition of cell proliferation, we analyzed the correlations between p-MET, c-MET, HGF, PTEN, EGFR, p-EGFR, and responsiveness to METi in all 14 brain tumor cell lines using both regression and Spearman correlation analyses as described in Materials and Methods. Responsiveness of cells to METi antisurvival or antiproliferative effects did not correlate with c-MET, p-MET, PTEN, EGFR, or p-EGFR expression levels (Fig. 3A and B). However, there was a statistically significant correlation between HGF expression levels and both METi-induced cell apoptosis and death (adjust $R^2 = 0.6357$, $P < 0.005$; Spearman correlation, $P = 0.005$) and METi-induced inhibition of cell proliferation (adjust $R^2 = 0.6563$, $P < 0.001$; Spearman correlation, $P < 0.001$; Fig. 3, Supplementary Table S1). These data show that HGF co-expression is an important determinant of responsiveness of brain tumor cells to c-MET inhibition.

**Pathway activation mapping of METi effects in high- and low-HGF–expressing cells**

To investigate the cell signaling pathway(s) that might predict and mediate the responses to METi, we conducted a pathway activation mapping analysis using reverse-phase protein microarrays. Phosphorylation/activation mapping by reverse-phase protein microarray was obtained after 24-hour treatment with METi (300 nmol/L) or vehicle on 3 cell lines that comprised the top quartile of the highest HGF expression (U1242, U87, A172) and on 4 cell lines that
comprised the bottom quartile of HGF expression (ONS76, DAOY, GBM-10, and T98G). Of the key signaling proteins measured, statistically significant differences between the effects of c-MET inhibitor treatment (calculated as the difference between triplicate independent experiments of the treatment – control/untreated) were found for 5 biochemically interconnected ERK pathway–linked signaling proteins that are found downstream of the c-MET receptor: JAK (Y1022/Y1023), \( P = 0.04 \); ERK1/2 (T202/Y204), \( P = 0.01 \); p53 (S15), \( P = 0.06 \); ELK (S383), \( P = 0.03 \); and RSK3 (T356/S360), \( P = 0.05 \) (Fig. 4; Supplementary Table S2). These data reveal a molecular activation signature that differentiates the responses to METi of high-HGF from low HGF cells and provide new insights into the pathways that mediate the effects of c-MET inhibition in METi-responsive tumors.

**The in vivo antitumor effects of METi are significantly greater in high-HGF–expressing glioblastoma xenografts than in low-HGF–expressing xenografts**

To determine whether HGF expression also predicts sensitivity to METi in vivo, we selected 2 cell lines that are known to generate in vivo glioblastoma xenografts in immunodeficient mice. One of the cell lines expresses high HGF (U87) and the other expresses low HGF (T98G). We generated xenografts from the 2 cell lines. One week after implantation, tumor formation was verified by MRI (not shown), and the animals were treated with oral gavage of METi (25 mg/kg body weight) or vehicle control every day for 3 weeks. The mice were euthanized 4 weeks after tumor implantation. Tumor sizes were measured on H&E-stained maximal brain cross-sectional areas using computer-assisted image analysis. METi significantly inhibited the

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**Figure 4.** Pathway activation mapping of c-MET inhibitor effects in high- and low-HGF–expressing cell lines. Cells were treated with METi or control and phosphorylation/activation mapping by reverse-phase protein microarray (RPMA) was obtained on 3 cell lines that comprised the top quartile of the highest HGF expression (U1242, U87, A172) and on 4 cell lines that comprised the bottom quartile of HGF expression (ONS-76, DAOY, GBM-10, and T98G). Statistically significant differences between the effects of c-METi treatment (calculated as the difference between triplicate independent experiments of the treatment – control/untreated) were found for 5 biochemically interconnected ERK pathway-linked signaling proteins that are found downstream of c-MET: JAK (Y1022/Y1023), \( P = 0.04 \); ERK1/2 (T202/Y204), \( P = 0.01 \); p53 (S15), \( P = 0.06 \); ELK (S383), \( P = 0.03 \); and RSK3 (T356/S360), \( P = 0.05 \). The figure reveals the biochemically interlinked signature of differential treatment response in the context of high versus low HGF production along with the RPMA data results as a histogram for each of the proteins.
**in vivo** growth of high-HGF–expressing U87-derived xenografts but did not affect the growth of low-HGF–expressing T98G-derived xenografts (Fig. 5A). Oral delivery of METi led to a decrease of 53% of U87 tumor volume (P < 0.01) but did not significantly alter the volume of T98G tumors (P = 0.64; Fig. 5B). These data show that HGF expression also predicts **in vivo** responsiveness of brain tumor xenografts to c-MET inhibition by METi. However, the application of the tumor volume measurements to clinically relevant animal survival endpoints is yet to be determined.

**Brief exogenous HGF/EGF treatment sensitizes glioblastoma cells and GSCs to RTK inhibitor–induced apoptosis**

While investigating the effects of METi on glioblastoma cell and GSC malignancy, we surprisingly noticed that METi induced significantly greater cell death when the cells were pretreated with HGF. We therefore systematically investigated this intriguing observation, also in response to EGFR kinase inhibition. Glioblastoma cells (U87, U373), primary glioblastoma cells (GBM10), and GSCs (1228, 0308) were pretreated with 20 ng/mL HGF for various times (2–24 hours) before treatment with METi for 24 hours. The cells were subsequently assessed for apoptosis using Annexin-V/7AAD flow cytometry. The results showed that METi induced significantly greater (34%–79%; 2-sample t test, P < 0.05) cell death in HGF pretreated cells than in control (Fig. 6A). The optimal time of preactivation that achieved greatest sensitization to METi-induced cell death was 2 to 6 hours. We observed a similar intriguing result when we pretreated glioma cells with EGF for 3 to 24 hours before treatment with the EGFR kinase inhibitor erlotinib. Erlotinib induced significantly greater cell death (45%–151%; 2-sample t test, P < 0.05) in EGF pretreated cells than in control (Fig. 6B). The optimal time of preactivation that achieved greatest sensitization of the receptor to erlotinib was 3 hours. These data suggest that cancer cells can be sensitized to c-MET and possibly also other RTK inhibitions by brief activation with their respective ligands.

**Brief exogenous HGF treatment sensitizes glioblastoma xenografts to METi in vivo**

On the basis of the above findings, we hypothesized that short-term exogenous activation of c-MET by HGF might sensitize glioblastoma in vivo xenograft growth to inhibition by METi. To test this hypothesis, we established GSC-derived xenografts in the flanks of immunodeficient mice. Flank xenografts were chosen to facilitate HGF delivery to the tumors as HGF protein might not penetrate the blood–brain barrier. The tumor-bearing animals were randomized and intratumorally injected with HGF at 400 ng/cm³ tumor volume or control for 2 hours before intratumoral injection of METi at 50 μmol/L/cm³ tumor volume. The animals were treated every day for 2 weeks, and tumor sizes and weights were measured. The results showed that METi induced moderately but statistically significantly greater inhibition of growth in HGF pretreated tumors than control (2-sample t test, P = 0.002; Wilcoxon test, P = 0.004; Fig. 6C and Figure 5. The **in vivo** antitumor effects of METi are significantly greater in high-HGF–expressing glioblastoma xenografts than in low-expressing xenografts. Glioblastoma cells U87 with high HGF expression and T98G with low HGF expression were stereotactically implanted in the striatum of immunodeficient mice (n = 6 for each treatment group). METi or vehicle control was administered by daily oral gavage starting 1 week after tumor implantation. The animals were euthanized 4 weeks after tumor implantation, and the volumes of all tumors were measured. The results show that METi significantly inhibited the growth of U87 xenografts but not the growth of T98G xenografts. A, representative brain cross-sections with implanted xenografts (arrows). B, quantification of tumor sizes. *P < 0.05.
Supplementary Fig.). These data suggest that short-term bolus pretreatment of tumors with HGF could be used as a novel strategy to improve the efficacy of c-MET kinase inhibitors.

Discussion

PF-2341066 (METi) is an orally bioavailable small-molecule kinase inhibitor of c-MET that has been tested in few experimental cancers (13, 38, 39) but not in brain tumors before. The factors that determine sensitivity of cancers to this drug have not been determined to date. We show that while METi strongly inhibits c-MET phosphorylation in brain tumor cells, the METi effects on cell proliferation and apoptosis vary tremendously between the different cells. We identify HGF as one key determinant of responsiveness to METi. Our data therefore suggest that c-MET kinase inhibitors are likely to achieve greater clinical benefits in patients with tumors expressing higher levels of HGF.
although these findings remain to be validated using animal survival studies to complement the tumor volume measurements. Surprisingly, responsiveness to METi did not correlate with p-MET which is usually induced by HGF. A probable explanation for this apparent inconsistency (illustrated by the findings in the ONS-76 cell line) is the previously described HGF-independent c-MET activation via mechanisms other than HGF and including basal c-MET kinase activity that increases with c-MET expression (40) and ligand-independent activation via dimerization with EGFR (41). While our manuscript was being prepared for submission, a publication by Xie and colleagues showed that c-MET autocrine loop predicts responsiveness to another c-MET kinase inhibitor (SGX523) (42). These findings, which were derived from fewer cell lines and which used an inhibitor that was retracted from a phase I clinical trial due to toxicity, are in line with our findings. On the other hand, a previous publication concluded that amplification of c-MET may identify a subset of rare gastric cancers with sensitivity to the selective tyrosine kinase inhibitor PHA-665752 (43). However, c-MET amplifications are relatively rare in brain tumors and were not detected in our cell lines. The c-MET inhibitor METi (PF-2341066) also has affinity to the anaplastic lymphoma kinase (ALK). However, while c-MET is a well-characterized oncogene in brain tumors (1, 2), ALK has not been described as a major regulator of these tumors. Therefore, the effects of METi in brain tumors are most likely due to inhibition of c-MET.

We also investigated the molecular signaling events that underlie responsiveness to c-MET inhibition in high-versus low-HGF–expressing cells. We found that p-p53 is significantly increased, whereas p-JAK, p-ERK, p-ELK, and p-RSK3 are significantly inhibited by METi in high-HGF–expressing cells but not in low-HGF–expressing cells. These data suggest that autocrine-activated c-MET depends on these molecules to maintain tumor cell proliferation and survival. The activation and stabilization of p53 are mediated by specific protein modifications, with phosphorylation at serine Ser-15 representing a critical event (44). JAK, ERK, ELK, and RSK3 are activated by c-MET and mediate its effects on proliferation, survival, and mitogenesis (45–48). The data provide insights into the molecular basis of responsiveness to c-MET inhibition in cancer cells.

Surprisingly, we also found that when glioblastoma cells and xenografts were pretreated with a pulse of exogenous HGF, METi induced greater cell apoptosis and tumor growth inhibition than in nonpretreated cells. We also observed this phenomenon for EGF/EGFR, suggesting that it might also occur in other RTKs. A possible explanation for this intriguing phenomenon is that short-term exogenous activation of c-MET by HGF triggers fast oncogene addiction that renders tumors dependent on c-MET (49). Another potential explanation is that ligand binding to the receptor induces conformational changes that render the receptor more susceptible to the inhibitor. A third possible explanation is based on the finding that the extracellular domain of c-MET interacts with the death receptor Fas to prevent Fas ligand (FasL) binding, Fas activation, and apoptosis through the extrinsic pathway (50). Exogenous HGF might therefore disrupt the Fas/MET aggregation leading to Fas activation and apoptosis. Irrespective of the underlying mechanism(s), our findings suggest that short-term delivery of ligands before treatment with RTK inhibitors might be developed as a new strategy for improving the efficacy of RTK inhibitors. Unlike long-term treatment, short-term delivery of ligands is not likely to enhance tumor growth as evidenced by our in vivo findings. Delivery of the ligand to brain tumors would probably be local due to questionable penetration of the blood–brain barrier by HGF.

Altogether, our findings suggest that c-MET activation by either long-term autocrine HGF/c-MET loop formation or short-term exogenous HGF application sensitizes cancer cells to c-MET kinase inhibition and that this might also apply for other RTKs.

Disclosure of Potential Conflicts of Interest
J. Christensen is employed by Pfizer as Sr. Director, Precision Medicine, Oncology Research Unit, and has ownership interest (including patents) in Pfizer, Inc. and is a patent holder on PF-2341066. No potential conflicts of interest were disclosed by the other authors.

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