Preclinical Development

Sensitivity of Selected Human Tumor Models to PF-04217903, a Novel Selective c-Met Kinase Inhibitor

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Abstract
The c-Met pathway has been implicated in a variety of human cancers for its critical role in tumor growth, invasion, and metastasis. PF-04217903 is a novel ATP-competitive small-molecule inhibitor of c-Met kinase. PF-04217903 showed more than 1,000-fold selectivity for c-Met compared with more than 150 kinases, making it one of the most selective c-Met inhibitors described to date. PF-04217903 inhibited tumor cell proliferation, survival, migration/invasion in MET-amplified cell lines in vitro, and showed marked antitumor activity in tumor models harboring either MET gene amplification or a hepatocyte growth factor (HGF)/c-Met autocrine loop at well-tolerated dose levels in vivo. Antitumor efficacy of PF-04217903 was dose-dependent and showed a strong correlation with inhibition of c-Met phosphorylation, downstream signaling, and tumor cell proliferation/survival. In human xenograft models that express relatively high levels of c-Met, complete inhibition of c-Met activity by PF-04217903 only led to partial tumor growth inhibition (38%–46%) in vivo. The combination of PF-04217903 with Recepteur d’origine nantais (RON) short hairpin RNA (shRNA) knockdown in the HT29 model that also expresses activated RON kinase–induced tumor cell apoptosis and resulted in enhanced antitumor efficacy (77%) compared with either PF-04217903 (38%) or RON shRNA alone (56%). PF-04217903 also showed potent antiangiogenic properties in vitro and in vivo. Furthermore, PF-04217903 strongly induced phospho-PDGFRβ (platelet-derived growth factor receptor) levels in U87MG xenograft tumors, indicating a possible oncogene switching mechanism in tumor cell signaling as a potential resistance mechanism that might compromise tumor responses to c-Met inhibitors. Collectively, these results show the use of highly selective inhibition of c-Met and provide insight toward targeting tumors exhibiting different mechanisms of c-Met dysregulation. Mol Cancer Ther; 11(4); 1036-47. ©2012 AACR.

Introduction
An extensive body of literature indicates that c-Met is one of the most frequently genetically altered or otherwise abnormally activated receptor tyrosine kinases (RTK) in various advanced human cancers (1, 2). The recent clinical results of MetMAB (c-Met monoclonal antibody) in combination with erlotinib showed improved progression-free survival and overall survival in patients with non–small cell lung carcinomas (NSCLC) with high c-Met expression (3), which provided clinical evidence for the benefit of targeting c-Met in cancer therapy.

c-Met is the prototypical member of a subfamily of RTKs, which also includes Recepteur d’origine nantais (RON) kinase. Hepatocyte growth factor (HGF), also known as scatter factor, is the only known high-affinity ligand of c-Met (4). Under physiologic conditions, c-Met is mainly activated by its ligand, HGF, in a paracrine fashion (4, 5) and is tightly regulated by ligand concentration and activation at the target cell surface and ligand-activated receptor internalization and degradation. Activation of the c-Met pathway provides a powerful signal for cell survival, migration, and proliferation (invasive growth), which is critical during embryonic development and wound healing (6, 7).

It is now widely accepted that the c-Met signaling that nourishes the early development and tissue regeneration is aberrantly activated in cancer cells and can lead to the neoplastic dissemination of tumors (7, 8). The propagation of c-Met–dependent invasive growth signals has been shown to be a general and important feature of highly aggressive tumors (9, 10). In most cancers, c-Met and HGF are overexpressed relative to surrounding tissues, and their expression correlates with poor patient prognosis (11, 12). In these cases, aberrant c-Met activation is...
reported to occur through transcriptional upregulation by various mechanisms including (i) other oncopines such as Ras (13) and β-catenin (14), (ii) environmental conditions such as hypoxia (15), or (iii) molecules generated by the reactive stroma of full-bloomed tumors such as inflammatory cytokines, growth factors (including HGF itself), and proangiogenic factors (16–18). In many of these tumors, c-Met activation is a secondary event—the consequence rather than the cause of the malignant phenotype and it serves as a co-activated oncopine that further exacerbates the malignant properties of already transformed cells by conveying its antiapoptotic, proliferative, angiogenic, and unique proinvasive signals to support tumor cells in overcoming selective pressures during cancer metastatic progression (19, 20).

In contrast to its role in promoting tumor invasive growth and metastasis in a wide range of cancers, a small subset of cancers where MET gene amplification has been identified became addicted to c-Met pathway activation. Amplification of the MET gene locus, with consequent protein overexpression and constitutive kinase activation, has been reported in patients with gastric, metastatic colorectal cancer, esophageal adenocarcinoma, medulloblastoma (21–24), and NSCLC with acquired resistance to EGFR receptor (EGFR) inhibitors (25). In these tumor models, c-Met inhibition results in a proliferative blockage and/or marked cell death and subsequent tumor regression (26–28).

Other mechanisms of c-Met mediated oncogenic pathway activation include MET gene mutation and aberrant autocrine ligand production. c-Met–activating point mutations in the kinase domain are implicated as the cause of hereditary papillary renal carcinoma and were also detected in sporadic papillary renal carcinoma, lung cancers, head and neck cancers, childhood hepatocellular carcinoma, and gastric cancer (29–33). Aberrant activation of c-Met by expressing both HGF and c-Met (HGF/c-Met autocrine loop formation) in tumor cells was also described for glioblastomas, breast carcinomas, rhabdomyosarcomas, and osteosarcomas (34–37). Furthermore, HGF/c-Met has been implicated in the regulation of tumor angiogenesis through the direct proangiogenic properties in endothelial cells or through regulation of secretion of angiogenic factors including VEGFA, interleukin (IL)–8, and thrombospondin-1 (38, 39).

Here, we describe PF-04217903, a potent and selective ATP-competitive inhibitor of c-Met, and its efficacy in tumor models where c-Met is activated by different mechanisms including c-Met gene amplification, HGF/c-Met autocrine loop formation, and c-Met overexpression. The unique feature of PF-04217903 is its exquisite selectivity compared with the numerous c-Met small-molecule inhibitors described to date. Lacking the confounding issue of off-target kinase inhibition, PF-04217903 is ideally suited for use as a tool inhibitor in preclinical models to dissect the role of c-Met catalytic activity in cancer progression.

Materials and Methods

**Compound**

PF-04217903, 2-{4-[3-quinolin-6-ylmethyl-3H]-[1,2,3]triazol-4,5-b]pyrazin-5-yl]-pyrazol-1-yl}-ethanol (Supplementary Fig. S1), was synthesized at Pfizer Global Research and Development, La Jolla Laboratories.

**Biochemical kinase assays**

c-Met catalytic activity was quantified using a continuous-coupled spectrophotometric assay in which the time-dependent production of ADP by c-Met was determined by analysis of the rate of consumption of NADH. NADH has a measurable absorbance at 340 nm and its consumption was measured by a decrease in absorbance at 340 nm as measured by spectrophotometry at designated time points. To determine Kᵢ values, PF-04217903 was introduced into test wells at various concentrations in the presence of assay reagents and incubated for 10 minutes at 37°C. The assay was initiated by addition of the c-Met enzyme.

**Cells**

Unless otherwise mentioned, cells were acquired from American Type Culture Collection. GTL-16 gastric carcinoma cells were a gift from Dr. Paolo Comoglio (University Torino Medical School, Candiolo, Italy). Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics, Inc.

**Cellular kinase phosphorylation ELISA assays**

Cells were seeded in 96-well plates in media supplemented with 10% FBS and transferred to serum-free media with 0.04% bovine serum albumin (BSA) after 24 hours. In experiments investigating ligand-dependent RTK phosphorylation, corresponding growth factors were added for up to 20 minutes. After incubation of cells with PF-04217903 for 1 hour and/or appropriate ligands, protein lysates were generated from cells. Total tyrosine phosphorylation of selected protein kinases was assessed by the standard sandwich ELISA method.

**Cell proliferation/survival assays**

Tumor cells were seeded in 96-well plates in the growth media (10% FBS) overnight for attachment. Media were replaced with serum-free media (0.04% BSA) and appropriate controls or designated concentrations of PF-04217903 were added. Cells were incubated at 37°C for 24 to 72 hours. HUVECs (passage 3) were grown to confluence in EGM2 media (Walkersville). Cells were seeded in EGM2 and incubated for 5 to 6 hours. After attachment, cells were cultured in serum-free media (Cell Applications) overnight at 37°C and treated with PF-04217903 and HGF (100 ng/mL) and incubated at 37°C for 48 to 72 hours. MTT (Promega) or resazurin (Sigma) assays were conducted to determine the relative cell numbers.
Tumor cell migration and Matrigel invasion assays
NCI-H441 cell and HUVEC migration/invasion were determined using the ACEA RT-CIM System (ACEA Biosciences) following the manufacturer’s instructions.

Apoptosis assay
GTL-16 cells were seeded in 96-well plates at 40,000 cells per well. Designated concentrations of PF-04217903 or vehicle were added to the wells in serum-free media. Cells were incubated in 37°C, 5% CO2 for 48 hours. The ssDNA Apoptosis ELISA Kit (Chemicon International) was used following manufacturer’s instructions.

In vivo studies
Animals. Female nu/nu mice were obtained from Charles River. Animals were maintained under clean room conditions in sterile filter top cages with Alpha-Dri bedding and housed on high-efficiency particulate air (HEPA)-filtered ventilated racks. Animals received sterile rodent chow and water ad libitum. All of the procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with Pfizer Animal Care and Use Committee guidelines.

Subcutaneous xenograft models in athymic mice. Tumor cells were implanted subcutaneously into the right flank region of each mouse and allowed to grow to the designated size. The athymic mice bearing established tumors were administered PF-04217903 either by oral gavage in 0.5% methylcellulose suspension or by implanting a mini Alzet-pump carrying the drug solution. Tumor volume was measured using electronic digital calipers. Percent (%) inhibition values were calculated as: 100 × (1 – [(treated final day – treated final day1)/control final day – control final day1)]. Tumor volumes were analyzed using one-way ANOVA. At the end of study, mice were humanely euthanized and tumors were resected. Proteins were extracted from the tumor samples and protein concentrations were determined using a BSA assay (Pierce). The level of proteins of interest in the tumor sample was determined using a capture ELISA method or immunoblotting.

Plasma human VEGFA and IL-8 assay. Athymic mice bearing established (150–800 mm3) xenografts were administered PF-04217903 in 0.5% methylcellulose suspension by oral gavage as multiple oral doses at the designated dose levels. At designated times following PF-04217903 administration, the serum samples were collected. The serum human VEGFA and IL-8 levels were measured by using the human VEGFA and IL-8 ELISA Kits from R & D Systems.

Results
PF-04217903 potently inhibited the catalytic activity of c-Met and its oncogenic mutants in vitro
PF-04217903 (structure in Supplementary Fig. S1) was identified as a potent ATP-competitive inhibitor of the catalytic activity of recombinant, human c-Met kinase with a mean IC50 of 4.8 nmol/L. In cell-based assays, PF-04217903 inhibited HGF-stimulated or constitutive total tyrosine phosphorylation of wild type c-Met with a mean IC50 value of 7.3 nmol/L across a panel of human tumor and endothelial cell lines (Supplementary Table S1) including GTL-16, H1993, and HT29 cells as shown in Fig. 1A. PF-04217903 also showed similar potency for inhibition of c-Met phosphorylation in mIMCD3 mouse epithelial cells (IC50 = 6.9 nmol/L) and MDCK canine kidney epithelial cells (IC50 = 9.5 nmol/L). As we reported previously (40), PF-04217903 also exhibited inhibitory activities against certain c-Met-activating mutations including V1092I (IC50 = 16 nmol/L), H1094R (IC50 = 3.1 nmol/L), M1250T (IC50 = 24 nmol/L), R988C (IC50 = 6.4 nmol/L), and T1101I (IC50 = 6.7 nmol/L), but not active against c-Met activation loop mutants Y1230C (IC50 > 10,000 nmol/L) and Y1235D (IC50 = 139 nmol/L).

PF-04217903 showed exquisite kinase selectivity
PF-04217903 was evaluated against a panel of more than 150 kinases in biochemical assays conducted at Upstate Inc. (124 kinases), University of Dundee (Dundee, UK; 40 kinases), and Pfizer (40 kinases). In these screening assays, PF-04217903 was determined to be greater than 1,000-fold selective for c-Met compared with each of the other kinases included in this collective kinase panel (40). In cell-based studies, PF-04217903 did not show inhibitory activity against closely related RTKs with IC50 selectivity ratio more than 1370, including RON, insulin receptor kinase, insulin-like growth factor 1 receptor (IGF1R), ROS1, or anaplastic lymphoma kinase (ALK) at concentrations up to 10 μmol/L (Supplementary Table S2). On the basis of these data, PF-04217903 showed exquisite selectivity for c-Met compared with a diverse set of more than 150 kinases representing one third of the known kinases. These data suggest that the pharmacologic activity of PF-04217903 observed in the present studies is likely mediated by c-Met as opposed to other nontarget kinases.

PF-04217903 efficacy against neoplastic phenotypes of cancer cells in vitro
c-Met has been shown to regulate cell growth, migration, and invasion of tumor cells (1). To evaluate the effect of PF-04217903 for its phenotypic effects, we selected a panel of tumor models that represent different types of aberrant c-Met upregulation. These models include GTL-16 human gastric carcinoma and H1993 human NSCLC with MET gene amplification, U87MG human glioblastoma with HGF/c-Met autocrine loop formation (36), and a panel of tumor cell lines that overexpress c-Met determined by fluorescence-activated cell-sorting (FACS) and Western blot analysis (Supplementary Fig. S2), such as HT29, Colo205, SW620 colon carcinomas, MDA-MB-231 breast carcinoma, and H292 NSCLC.
PF-04217903 inhibited proliferation of c-Met–amplified human GTL-16 gastric carcinoma and H1993 NSCLC cells with IC_{50} values of 12 and 30 nmol/L, respectively (Fig. 1B and Table 1) and induced apoptosis of GTL-16 cells (IC_{50} = 31 nmol/L; Fig. 1C and Table 1). The strong correlation of IC_{50} values for inhibition of c-Met phosphorylation (Fig. 1A) and c-Met–dependent phenotypes in these cells (Fig. 1B–D) suggests that PF-04217903 pharmacologic activity is mediated by inhibiting c-Met catalytic activity. In contrast, despite the potent inhibition of Met activity by PF-04217903 in the cell lines that either harbor HGF/c-Met autocrine loop or overexpress c-Met (Fig. 1A; Supplementary Table S1), it only partially inhibited HGF-mediated cell proliferation in these cells, as represented by U87MG human glioblastoma or SW620 and HT29 colon carcinoma (Fig. 1B) as well as by additional cell lines shown in Table 1 (IC_{50} values > 10 μmol/L). PF-04217903 also inhibited HGF-mediated cell migration and Matrigel invasion in several c-Met–overexpressing tumor cell lines such as human NCI-H441 lung carcinoma (Fig. 1D) and HT29 colon carcinoma with IC_{50} values comparable with those for inhibition of c-Met phosphorylation in these cell lines (IC_{50} = 7–12.5 nmol/L; Table 1).

PF-04217903 antitumor efficacy and relationship between inhibition in c-Met phosphorylation and tumor growth in c-Met–amplified GTL-16 model

GTL-16 tumors harboring MET gene amplification were used to evaluate PF-04217903 antitumor efficacy and the relationship of c-Met target inhibition to tumor growth inhibition (TGI) in this category of tumors in vivo. In this study, PF-04217903 showed dose-dependent TGI (Fig. 2B), which correlated with the inhibition in c-Met phosphorylation in these tumors (Fig. 2A). To further investigate the relationships between PF-04217903 steady-state plasma exposure (or C_{min}), c-Met phosphorylation, and antitumor efficacy, we conducted a pump infusion study. Alzet osmotic minipumps were implanted subcutaneously in mice bearing established GTL-16 tumors and were primed to deliver various concentrations of PF-04217903 at dose levels of 0.15, 0.5, 1.5, 5, or 15 mg/kg/d for a period of 14 days. Tumor volumes were measured throughout the study. Plasma and tumor tissues were collected at the end of the study for the assessment of plasma drug concentration and tumor biomarkers. Similar to the results in the oral dosing study, a concentration-dependent inhibition of c-Met phosphorylation and antitumor efficacy were also observed in the infusion study (Fig. 2C).

Figure 1. Effect of PF-04217903 on Met phosphorylation in GTL-16, NCI-H1993, and HT29 cells (A); cell proliferation in GTL-16, NCI-H1993, U87MG, SW620, and HT29 cells (B); GTL-16 cell apoptosis (C); and NCI-H441 cell Matrigel invasion (D). A, the cells were seeded in the 96-well plates and were treated with designated concentrations of PF-04217903 for 1 hour. Capture ELISA assay was conducted to determine Met phosphorylation status. B, the cells were seeded in low density in the 96-well plates and were treated with designated concentrations of PF-04217903. U87MG, SW620, and HT29 cells were also treated with HGF (20 ng/mL). MTT or resazurin assays were conducted 72 hours later to assess the number of living cells. C, GTL-16 cells were treated with PF-04217903 for 24 hours in growth media (RPMI + 10% FBS), and cell apoptosis was detected by using the ssDNA Apoptosis ELISA Kit (Chemicon International). The ssDNA content of the cells was quantified as percentage of control cells. D, NCI-H441 cells were treated with HGF (25 ng/mL) or designated concentrations of PF-04217903. Migration and invasion of the cells across a membrane and a layer of Matrigel were assessed real-time for 48 hours by the electronic sensor embedded in the bottom of the well using AECI CIM Cell System. Invaded cells were also fixed and stained with Diff-Quick Fixative Solution and imaged by using the ImagePro Imaging System.
and D). The pharmacokinetic and pharmacodynamic relationship of TGI and c-Met target modulation versus free plasma drug concentration is illustrated by Hill function plots in Fig. 2D. The following parameters for PF-04217903 were calculated for the infusion study: (i) inhibition in c-Met phosphorylation: EC50 = 10 nmol/L and EC90 = 45 nmol/L, (ii) TGI: EC50 = 13 nmol/L and EC90 = 80 nmol/L, and (iii) the relationship between c-Met target inhibition and tumor growth: 90% of c-Met inhibition correlated with 80% TGI. Collectively, these studies established the following conclusions about targeting c-Met in the GTL-16 model that harbors Met gene amplification: (i) the extent and duration of c-Met inhibition was directly linked to the level of antitumor efficacy, (ii) near-complete inhibition of c-Met (>90%) during the entire treatment period was necessary to achieve robust antitumor effects.

To investigate the mechanisms of antitumor efficacy in GTL-16 tumors, PF-04217903 was evaluated for its effects on c-Met–mediated signal transduction and effect on tumor cell function in tumor tissues. PF-04217903 showed dose-dependent inhibition of critical signal transduction events downstream of c-Met including Gab-1, AKT, extracellular signal-regulated kinase (Erk), PLC\textsubscript{1}, and STAT5 as shown by Western blot analysis (Fig. 2F). A dose-dependent decrease in the number of Ki67-positive cells was observed following immunohistochemical analysis of tumor sections taken at day 16 of PF-04217903 treatment at 3, 10, and 30 mg/kg/d in the GTL-16 model (Fig. 2G), which correlated with the inhibition of c-Met phosphorylation (Fig. 2E). Furthermore, Western blot analysis showed a dose-dependent induction of activated caspase-3 levels at 3, 10, and 30 mg/kg/d in the same GTL-16 study (Fig. 2H).

Antitumor efficacy of PF-04217903 in U87MG model exhibiting a HGF/c-Met autocrine loop

HGF/c-Met autocrine loop formation has been identified in several types of human cancers such as glioblastoma and breast carcinoma and predicts an invasive phenotype (41). The U87MG human glioblastoma model expresses both HGF and c-Met and was selected to evaluate PF-04217903 in an autocrine setting. In contrast to the minimal effect observed in U87MG cell growth in vitro, significant TGI was achieved in all 5 dose groups, with 10 and 30 mg/kg dose levels showing marked tumor size reduction of 68% and 84%, respectively (Fig. 3A). This dose-dependent TGI correlated with the decreased Ki67 expression in these tumors determined by immunohistochemical analysis (Fig. 3C). Phospho-RTK array analysis

### Table 1. Effect of PF-04217903 on c-Met–dependent cellular phenotypes in vitro

<table>
<thead>
<tr>
<th>Assay</th>
<th>Tumor cell phenotypes</th>
<th>c-Met status</th>
<th>PF-04217903 concentration IC50(^a) nmol/L</th>
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<tr>
<td>Tumor cell phenotypes</td>
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<tr>
<td>Proliferation (MTT assay) of GTL-16 gastric carcinoma cells (mean IC(_{50}))</td>
<td>Gene amplification</td>
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<tr>
<td>Proliferation (MTT assay) of NCI-H1993 lung carcinoma cells (mean IC(_{50}))</td>
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<td>Proliferation (MTT assay) of a panel of tumor cell lines(^b) (mean IC(_{50}))</td>
<td>Overexpression/autocrine</td>
<td>&gt;10,000</td>
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<tr>
<td>Apoptosis (ssDNA assay) of GTL-16 gastric carcinoma cells (mean IC(_{50}))</td>
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<td>HGF-stimulated HT29 cell (Boyden Chamber Matrigel invasion; mean IC(_{50}))</td>
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<tr>
<td>HGF-stimulated NCI-H441 cell migration (Acea System; IC(_{50}))</td>
<td>Overexpression</td>
<td>11</td>
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<td>HGF-stimulated NCI-H441 cell invasion (Acea System; IC(_{50}))</td>
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<td>12.5</td>
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<td>HGF-stimulated HUVEC Matrigel invasion (Acea System; IC(_{50}))</td>
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<td>Apoptosis (ssDNA assay) of HUVEC cells (IC(_{50}))</td>
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</table>

Abbreviation: ss, single-stranded.

\(^a\)IC\(_{50}\) values were generated by curve fitting using a 4-parameter analysis.

\(^b\)The panel of tumor cell lines includes Colo205, HT29, SW620, HCT116, DLD1, MDA-MB-231, MDA-MB-468, HCC1143, A549, U87MG, PC3, and others.
Figure 2. Inhibition of c-Met phosphorylation (A, D, E), tumor growth (B–D), c-Met downstream signal transduction (F), tumor cell proliferation (G), and induction of apoptosis (H) by PF-04217903 in GTL-16 xenograft model. Athymic mice bearing established GTL-16 tumors (250 mm³ in A, B, C, D, G, H) or 400 mm³ in E and F were administered PF-04217903 orally or subcutaneous minipump infusion at the indicated dose or vehicle alone over the designated treatment schedule. *, a significant difference from the control group (P < 0.05) as determined by one-way ANOVA.

A, dose-dependent inhibition of c-Met phosphorylation in GTL-16 xenograft tumors following 16 days of oral dosing. Phospho-c-Met levels were measured using ELISA. % inhibition of c-Met phosphorylation was calculated as: % inhibition = 100 – [mean treated/mean untreated] × 100. B, inhibition of GTL-16 tumor growth in athymic mice following 16 days of PF-04217903 oral dosing. C, inhibition of GTL-16 tumor growth in athymic mice following 14 days of subcutaneous pump infusion. D, the relationship of free plasma concentration of PF-04217903 to HGF/c-Met phosphorylation in tumors (% inhibition) and TGI (tumor growth inhibition) as determined using one-way ANOVA. Values = mean ± SEM, n = 6–12.

E, dose-dependent decrease in the number of Ki67-positive cells following 16-day drug treatment determined by Western blot analysis. G, dose-dependent decrease in the number of Ki67-positive cells following 14 days of treatment determined by Western blot analysis.

H, induction of apoptosis (cleaved caspase-3) following 16 days of PF-04217903 treatment in GTL-16 tumors determined by Western blot analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; QD, once daily.

showed that besides c-Met, EGFR and platelet-derived growth factor receptor (PDGFR)/β were also co-activated in U87MG xenograft tumors (data not shown). To investigate the mechanisms underlining the observed antitumor efficacy in this model, we conducted Western blot analysis with tumor lysates to assess the status of the 3 co-activated RTKs (c-Met, EGFR, and PDGFR) as well as RTK downstream signaling. As shown in Fig. 3B, 5 hours (Tₘₐₓ) after oral dosing, PF-04217903 dose dependently inhibited c-Met, Gab-1, Erk1/2, and AKT phosphorylation and induced apoptosis (cleaved caspase-3) in U87MG xenograft tumors at all dose levels (5, 15, and 50 mg/kg).
orally/once daily for 3 days). In addition, there was no significant change in phospho- or total EGFR levels associated with PF-04217903 treatment in this study, but interestingly a strong and dose-dependent induction in phospho-PDGFR\(_{\beta}\) levels were observed (Fig. 3D).

Antitumor efficacy of PF-04217903 in human xenograft models exhibiting c-Met overexpression

c-Met is highly expressed in almost every type of cancer (2). To investigate the relationship of c-Met overexpression in response to PF-04217903, we selected a panel of tumor cell lines that express high level of c-Met without \(MET\) gene amplification. SW480 and SW620 are 2 colon carcinoma cell lines derived from the same patient. SW480 was obtained from primary colon cancer tissue and SW620 was isolated 1 year later from a metastatic lymph node (42). FACS analysis of expression levels of c-Met and related RTKs showed that SW480 expressed relatively low c-Met protein and high EGFR, but SW620 expressed high c-Met and low EGFR (Fig. 4A). PF-04217903 administered orally at 40 mg/kg/d showed marked inhibition of c-Met phosphorylation in SW620 tumors for the entire treatment period (Fig. 4C) and resulted in a 45% TGI (Fig. 4B). The same dosing regimen of PF-04217903 in SW480 tumors had no effect on tumor growth (Fig. 4B), suggesting that elevated c-Met expression may also be an important factor for tumor response. Furthermore, following 27 days of PF-04217903 treatment, c-Met total protein levels in SW620 tumors increased significantly as shown by Western blot analysis (Fig. 4C). This increase in total c-Met protein expression did not associate with \(c-Met\) gene copy number increase determined by quantitative PCR analysis (data not shown).

The HT29 human colon carcinoma cell line expressed a relatively high level of c-Met and related RTKs (Fig. 3D) such as RON, as well as RON oncogenic variants—RON-\(D_{160}\) and sf-RON (43). PF-04217903, orally administered at 50 mg/kg/d, significantly inhibited c-Met phosphorylation (Fig. 4F) and showed a 40% decrease in HT29 tumor growth (Fig. 4E). To investigate whether RON also contributes to progression of HT29 tumors, a RON short hairpin RNA (shRNA) was used alone and in combination with PF-04217903 treatment. Combining PF-04217903...
with RON shRNA achieved an enhanced antitumor efficacy (77%) compared with either PF-04217903 (40%) or RON shRNA alone (52%; Fig. 4E). Western blot analysis indicated that (i) PF-04217903 monotherapy increased phospho-AKT levels and did not induce tumor apoptosis, (ii) combining PF-04217903 with RON shRNA knocked down blocked phospho-AKT upregulation observed in PF-04217903 monotherapy, (iii) Ron shRNA knockdown significantly increased activated caspase-3 levels compared with the HT29 vector control tumors with or without PF-04217903 treatment (Fig. 4E). PF-04217903 also partially inhibited tumor growth of Colo205 human colon carcinoma, MDA-MB-231 human breast carcinoma, and H292 human NSCLC xenograft
tumors, that overexpress c-Met, by 44%, 43%, and 39%, respectively (Fig. 4G–I). Collectively, these results indicate that near-complete inhibition of c-Met activity by PF-04217903 in the selected c-Met-overexpressing models resulted in significant but incomplete TGI. PF-04217903 was well tolerated in mice. There was no animal body weight loss observed in any of the dose levels tested in the in vivo studies.

**Antiangiogenic effects of PF-04217903 in vitro and in vivo**

HGF/c-Met pathway is known to promote tumor angiogenesis therefore fostering cancer progression (1, 2, 41). PF-04217903 was evaluated for its antiangiogenic activity in vitro and in vivo. PF-04217903 inhibited HGF-stimulated c-Met phosphorylation in HUVECs with an IC₅₀ value of 4.6 nmol/L (Supplementary Table S1). In endothelial cell functional assays, PF-04217903 inhibited HGF-mediated HUVEC survival (IC₅₀ = 12 nmol/L), Matrigel invasion (IC₅₀ = 27 nmol/L), and induced HUVEC apoptosis (IC₅₀ = 7 nmol/L; Table 1). PF-04217903 was evaluated for modulation of microvessel density (MVD) assessed by immunostaining for CD31 (platelet endothelial cell adhesion molecule 1) in vivo. A significant reduction of CD31-positive endothelial cells was observed at 3, 10, and 30 mg/kg/d in U87MG tumors following 10 days of PF-04217903 treatment (Fig. 5A), indicating that inhibition of MVD correlated with significant antitumor efficacy (Fig. 3A). c-Met and HGF have also been shown to regulate the secretion of proangiogenic factors, including VEGFA and IL-8 by tumor cells (16–18). Therefore, the effect of PF-04217903 on VEGFA and IL-8 plasma levels was assessed. In these studies, PF-04217903 showed a significant dose-dependent reduction of human IL-8 levels in both the U87MG and GTL-16 models (Fig. 5B and C) and decreased human VEGFA levels in the GTL-16 model (Fig. 5C). These data suggest that the antiangiogenic activity of PF-04217903 could be mediated either through direct effect in endothelial cells and/or through reduced secretion of proangiogenic factors by tumor cells.

**Discussion**

In this report, we described a novel ATP-competitive, potent, and highly selective small-molecule inhibitor of c-Met. The antitumor efficacy of PF-04217903 was also evaluated in tumor models that (i) harbor MET gene amplification, (ii) exhibit an HGF/c-Met autocrine loop, or (iii) express high levels of c-Met. Because it is highly...
selective against c-Met, PF-04217903 is an ideal tool to study the consequences of specifically perturbing c-Met catalytic activity in various cancer models without the perplexing effects of off-target kinase inhibition. Therefore, the antitumor efficacy following PF-04217903 treatment is most likely exclusively mediated by inhibiting c-Met activity in the tumor models tested.

Oncogene addiction is a phenomenon where some tumors become dependent on a single overactive oncogene and its intracellular pathways for their growth and survival due to gene mutation or amplification or rearrangement, and pharmacologic inhibition of this oncogene leads to tumor growth arrest and cell death (44). The robust antiproliferation and ant-survival effects we observed upon inhibiting c-Met catalytic activity by PF-04217903 in MET-amplified GTL-16 model are similar to that observed in EGFR-addicted or ALK fusion cell lines upon treatment with EGFR or ALK inhibitors (45, 46). These results support the notion that MET amplification can lead to a c-Met addicted tumor type or patient population that would likely respond to c-Met inhibitor therapy. Furthermore, the studies elucidating the relationship between inhibition of c-Met phosphorylation and tumor growth following PF-04217903 administration in the GTL-16 model show that near-complete inhibition (>90%) of c-Met activity during the entire dosing period is necessary to maximize therapeutic benefit. These preclinical findings provided the pharmacologic bases for estimation of PF-04217903 clinical dose and efficacious plasma concentrations.

High HGF levels have been identified in glioblastomas (47). Both glioblastoma cells and the associated neural endothelial cells were found to express HGF and c-Met. The interactions among the tumor and stroma cells through HGF-dependent autocrine and paracrine stimulation contribute to the proliferative and angiogenic phenotypes of gliomas (48). PF-04217903 produced a robust dose-dependent TGI and tumor cell apoptosis in the U87MG xenograft model, which is similar to that observed in the MET-amplified GTL-16 model. In contrast, PF-04217903 only showed minimal antiproliferative effect in U87MG cells in vitro. This discrepancy might be due to the absence of tumor microenvironment in the in vitro setting. PF-04217903 induced endothelial cell apoptosis in vitro and reduced MVD in U87MG tumors as well as decreasing the serum level of IL-8 in the U87MG tumor-bearing mice. These findings indicate that the in vivo efficacy observed in U87MG model could also be mediated by the antiangiogenic effect of PF-04217903 through direct inhibition of endothelial cell survival or indirect inhibition of angiogenic cytokine secretion by U87MG cells. Shojaei and colleagues reported that HGF/c-Met acted as an alternative angiogenic pathway in sunitinib-resistant tumors (49), providing additional evidence to support the role of HGF/c-Met in tumor angiogenesis.

We observed the co-activation of c-Met, EGFR, and PDGFRβ in U87MG xenograft tumors. It has been exten-sively reported that these 3 RTKs and their corresponding ligands are frequently overexpressed in gliomas, which correlates with higher tumor grade (47). Following 3 days of oral administration of PF-04217903, we found no significant change in EGFR activity and expression in the U87MG model but surprisingly PDGFRβ was strongly activated in this study. This finding showed a potential escape mechanism of oncogene switching through the PDGFRβ pathway in cell signaling in response to c-Met pathway inhibition. Future studies combining PF-04217903 with a PDGFR inhibitor in the U87 model will be necessary to elucidate the significance of this finding. Interestingly, we also noticed that following PF-04217903 treatment, strong inhibition of Gab-1 activities only correlated with c-Met activity but not with EGFR or PDGFRβ activities, indicating a unique signaling cascade through the c-Met/Gab-1 axis in the U87MG tumors. di Tomaso and colleagues recently reported that increased c-Met and PDGFR expression level was observed in the patients with glioblastoma who escaped from anti-VEGF therapy (cediranib) indicating potential applications of combining anti-c-Met with antiangiogenic and/or anti-PDGFR therapies in glioblastomas (50).

c-Met overexpression has been identified in the majority of human cancers and represents the most common type of c-Met deregulation. In contrast to the robust antitumor efficacies observed in the GTL-16 and U87MG models, complete inhibition of c-Met phosphorylation by PF-04217903 only led to partial inhibition of tumor growth (38%–48%) in several xenograft models that overexpress c-Met, as well as in a panel of c-Met–overexpressing tumor cell lines in vitro where exogenous HGF was present. These findings raised the following questions: (i) Are these partial antitumor efficacies in c-Met–overexpressing preclinical models significant enough to translate into clinical activity? and (ii) Can c-Met overexpression serve as a predictive biomarker for patient response to c-Met inhibitor therapy? It is noted that besides c-Met overexpression, these tumor cell lines also possess other onco- genic alterations, such as the KrasG12D mutation in SW620, the BrafV600E mutations in HT29 and Colo205, and the KrasG13D in the triple-negative MDA-MB-231 breast carcinoma, indicating that c-Met overexpression and activation are most likely a secondary event—the consequence rather than the cause of the malignant phenotype in these cancers. Our observation of c-Met overexpression and activation only in the SW620 lymph node metastasis, but not in the SW480 primary colon cancer, illustrates this scenario. PF-04217903 reduced tumor growth in SW620 model (45%) but had no effect in the SW480 model, suggesting that c-Met overexpression might be required for tumor response. This observation is consistent with the recent clinical finding that MetMAB plus erlotinib showed improved progression-free survival and overall survival only in patients with NSCLC with high c-Met expression (51). Furthermore, RTK coactivation could also affect tumor response to targeted therapies (20, 51). Using phospho-RTK array analysis, we found that multiple
RTKs were concomitantly activated in these tumors such as EGFR, fibroblast growth factor receptor (FGFR3), and RON in HT29; FGFR3, IGF1R, RON, c-RET, and Tie2 in SW620 (data not shown). These data indicate that c-Met may play a cooperative role with other coactivated RTKs to sustain malignancy in these tumors. Therefore, the partial antitumor efficacy by PF-04217903 might be due to the sustained downstream signaling mediated by other coactivated RTKs, and combination approach with c-Met inhibitor and other targeted therapies might be necessary to maximize efficacy. In the HT29 model where both c-Met and RON/RON160/sf-RON were overexpressed and activated, we observed enhanced TGI by combining PF-04217903 with RON shRNA knockdown. The effect was additive as determined by fractional product analysis (data not shown). In this study, a partial efficacy (40%) by PF-04217903 alone corresponded to an incomplete mitogen-activated protein kinase (MAPK) inhibition and upregulated AKT activity, suggesting that a signaling escape mechanism through the AKT pathway might play a role for sustained cell survival. Combining PF-04217903 with RON shRNA significantly inhibited MAPK activity blocked PF-04217903–induced phospho-AKT upregulation and resulted in HT29 tumor apoptosis and an enhanced TGI (77%). These findings indicate that both c-Met and RON contributed to tumor progression in the HT29 model, and combining anti c-Met and RON therapy showed a therapeutic benefit of the combination approach for c-Met inhibitors in c-Met-overexpressing cancers. Because the HT29 tumor also expresses a high level of activated EGFR, we included a treatment group with erlotinib as a single agent in the same HT29 study and achieved 55% TGI (data not shown), which is similar to that observed with RON shRNA alone (52%), indicating that EGFR also played a partial role in sustaining HT29 tumor growth. Collectively, our results suggest that although c-Met overexpression alone is not a biomarker for c-Met oncogene addiction, the elevated c-Met level might be a prerequisite for response to c-Met inhibitors. Furthermore, combination approaches with c-Met inhibitors and other relevant targeted therapy or therapies will most likely result in a better therapeutic outcome than c-Met inhibitor single-agent therapy in the majority of cancers that overexpress activated c-Met.

In summary, our studies show the therapeutic potential of PF-04217903 in targeting c-Met as a single agent for c-Met addicted cancers or in combination with other therapeutic agents in the scenario where c-Met acts as a codriver in oncogene co-activated cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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