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

Helen Y. Zou1*, Qiuhua Li1 Joseph H. Lee1, Maria E. Arango2, Kristina Burgess1, Ming Qiu1, Lars D. Engstrom1, Shinji Yamazaki1, Max Parker1, Sergei Timofeevski1, Jingrong Jean Cui1, Michele McTigue1, Gerrit Los1, Steven L. Bender1, Tod Smeal, and James G. Christensen1*

1PGRD-La Jolla, Pfizer, Inc., 10724 Science Center Dr. San Diego, CA 92121; 2Sanford Burnham Medical Research Institute, Orlando, FL.

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To Whom requests for reprints and correspondence should be addressed:

Helen Y. Zou
Oncology Research Unit
Pfizer Global Research & Development, La Jolla Labs,
10724 Science Center Dr. La Jolla, CA 92121
Telephone: (858) 622-3154
E-mail: helen.zou@pfizer.com

James G. Christensen
Oncology Research Unit
Pfizer Global Research & Development, La Jolla Labs,
10724 Science Center Dr. La Jolla, CA 92121
Telephone: (858) 638-6336
E-mail: james.christensen@pfizer.com

Abbreviations: HGF, hepatocyte growth factor; SF, scatter factor; RTK, receptor tyrosine kinase; ALK, anaplastic lymphoma kinase; NSCLC, nonsmall cell lung cancer; RON, Recepteur d’Origne Nantais; EGFR, epidermal growth factor receptor; IRK, Insulin Receptor Kinase; ERK, extracellular regulated kinase; STAT, signal transducer and activator of transcription; PLC, phospholipase C; PI-3-Kinase/PI3K, phosphotidylinositol-3-kinase; HUVEC, human umbilical vein endothelial cells.
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-4217903 is a novel ATP-competitive small molecule inhibitor of c-Met kinase. PF-04217903 demonstrated >1000 fold selectivity for c-Met compared with >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 demonstrated marked antitumor activity in tumor models harboring either MET gene amplification or a HGF/c-Met autocrine loop at well-tolerated dose levels in vivo. Antitumor efficacy of PF-04217903 was dose-dependent and demonstrated a strong correlation with inhibition of c-Met phosphorylation, downstream signaling, 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 RON shRNA knockdown in the HT29 model that also expresses activated RON kinase induced tumor cell apoptosis and resulted in enhanced antitumor efficacy (77%) compared to either PF-04217903 (38%) or RON shRNA alone (56%). PF-04217903 also demonstrated potent anti-angiogenic properties in vitro and in vivo. Furthermore, PF-04217903 strongly induced phospho-PDGFRβ 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 demonstrate the utility of highly selective inhibition of c-Met and provide insight toward targeting tumors exhibiting different mechanisms of c-Met dysregulation.
**Introduction**

An extensive body of literature indicates that c-Met is one of the most frequently genetically altered or otherwise abnormally activated RTKs in various advanced human cancers (1-2). The recent clinical results of MetMAB (c-Met monoclonal antibody) in combination with erlotinib demonstrated improved progression free survival (PFS) and overall survival (OS) in NSCLC patients 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 (SF), is the only known high-affinity ligand of c-Met (4). Under physiological 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 1) other oncogenes such as Ras (13) and β-Catenin (14), 2) environmental conditions such as hypoxia (15) or 3) molecules generated by the reactive stroma of full-blown tumors such as inflammatory...
cytokines, growth factors (including HGF itself) and pro-angiogenic 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 oncogene that further exacerbates the malignant properties of already transformed cells by conveying its anti-apoptotic, proliferative, angiogenic and unique pro-invasive 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 become ‘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 non-small-cell lung carcinomas (NSCLC) with acquired resistance to epidermal growth factor 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, 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 \textit{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]triazolo[4,5-b]pyrazin-5-yl)-pyrazol-1-yl]-ethanol (Fig. S1) was synthesized at Pfizer Global Research and Development, La Jolla Laboratories.

**Biochemical Kinase Assays.** c-Met catalytic activity was quantified utilizing 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). HUVEC (human umbilical vein endothelial cells) cells were purchased from Clonetics, Inc.

**Cellular kinase phosphorylation ELISA assays.** Cells were seeded in 96-well plates in media supplemented with 10% fetal bovine serum (FBS) and transferred to serum-free media with 0.04% bovine serum albumin (BSA) after 24 h. In experiments investigating ligand-dependent RTK phosphorylation, corresponding growth factors were added for up to 20 min. After incubation of cells with PF-04217903 for 1 h 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 was 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 h. HUVEC cells (passage 3) were grown to confluence in EGM2 media (Walkersville). Cells were seeded in EGM2 and incubated for 5 to 6 h. 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 48-72 h. MTT (Promega) or Resazurin (Sigma) assays were performed 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 manufacture’s instructions.

**Apoptosis assay.** GTL-16 cells were seeded in 96 well plates at 40,000 cells/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 h. 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 (Wilmington, MA). Animals were maintained under clean room conditions in sterile filter top cages with Alpha-Dri bedding and housed on 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 SC 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_{Day\ 1}) / (Control_{Final\ day} – Control_{Day\ 1})]\}. Tumor volumes were analyzed using one-way analysis of variance. 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 utilizing 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, Supplementary Fig. S1) was identified as a potent ATP-competitive inhibitor of the catalytic activity of recombinant, human c-Met kinase with a mean Ki of 4.8 nM. In cell-based assays, PF-04217903 inhibited HGF-stimulated or constitutive total tyrosine phosphorylation of wild type c-Met with a mean IC$_{50}$ value of 7.3 nM across a panel of human tumor and endothelial cell lines (Supplementary Table 1) including GTL16, H1993 and HT29 cells as shown in Figure 1A. PF-04217903 also demonstrated similar potency for inhibition of c-Met phosphorylation in mIMCD3 mouse epithelial cells (IC$_{50}$ = 6.9 nM) and MDCK canine kidney epithelial cells (IC$_{50}$ = 9.5 nM). As we reported previously (40), PF-04217903 also exhibited inhibitory activities against certain c-Met activating mutations including V1092I (IC$_{50}$, 16 nM), H1094R (IC$_{50}$, 3.1 nM), M1250T (24nM), R988C (IC$_{50}$, 6.4 nM) and T11010I (IC$_{50}$, 6.7nM), but not active against c-Met activation loop mutants Y1230C (IC$_{50}$, >10,000 nM) and Y1235D (IC$_{50}$, 139 nM).

**PF-04217903 demonstrated exquisite kinase selectivity.**

PF-04217903 was evaluated against a panel of >150 kinases in biochemical assays performed at Upstate Inc. (124 kinases), University of Dundee (40 kinases), and Pfizer (40 kinases). In these screening assays, PF-04217903 was determined to be greater than 1000-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 demonstrate inhibitory activity against closely related RTKs with IC$_{50}$ selectivity ratio > 1370, including RON, Insulin Receptor Kinase (IRK), Insulin-like Growth Factor 1 Receptor (IGF1R), ROS1, or Anaplastic Lymphoma Kinase (ALK) at
concentrations up to 10 µM (Supplementary Table 2). Based on this data, PF-04217903 demonstrated exquisite selectivity for c-Met compared with a diverse set of >150 kinases representing a third of the known kinases. This data suggests that the pharmacologic activity of PF-04217903 observed in the present studies is likely mediated by c-Met as opposed to other non-target 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 FACS and Western blot analysis (Supplementary Fig 2), 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 IC50 values of 12 nM and 30 nM respectively (Fig 1B, Table 1), and induced apoptosis of GTL-16 cells (IC50 = 31 nM, Fig 1C, Table 1). The strong correlation of IC50 values for inhibition of c-Met phosphorylation (Fig 1A) and c-Met-dependent phenotypes in these cells (Figure 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 (Figure 1A, Supplementary Table 1), 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 >10uM). PF-04217903 also inhibited HGF mediated cell migration and matrigel invasion in several c-Met over-expressing tumor cell lines such as human NCI-H441 lung carcinoma (Fig 1D) and HT-29 colon carcinoma with IC$_{50}$ values comparable to those for inhibition in c-Met phosphorylation in these cell lines (IC$_{50}$ = 7-12.5 nM; Table1).

**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 demonstrated dose dependent TGI (Fig 2B), which correlated to the inhibition in c-Met phosphorylation in these tumors (Fig 2A). To further investigate the relationships between PF-04217903 steady-state plasma exposure (or Cmin), c-Met phosphorylation, and anti-tumor efficacy, we conducted a pump infusion study. Alzet osmotic mini-pumps 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/day 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 anti-tumor efficacy were also observed in the infusion study (Fig 2C-D). The pharmacokinetic and pharmacodynamic relationship of tumor growth inhibition and c-Met target modulation vs. free plasma drug concentration is illustrated by Hill function plots in Figure 2D. The following parameters for PF-04217903 were calculated for the infusion study: 1) inhibition in c-Met
phosphorylation: EC$_{50}$ = 10 nM and EC$_{90}$ = 45 nM, 2) tumor growth inhibition: EC$_{50}$ = 13 nM and EC$_{90}$ = 80 nM, 3) the relationship between c-Met target inhibition and tumor growth: 90% of c-Met inhibition correlated with 80% tumor growth inhibition. Collectively, these studies established the following conclusions regarding targeting c-Met in the GTL-16 model that harbors Met gene amplification: 1) the extent and duration of c-Met inhibition was directly linked to the level of anti-tumor efficacy, 2) near complete inhibition of c-Met (>90%) during the entire treatment period was necessary to achieve robust anti-tumor effects.

To investigate the mechanisms of antitumor efficacy in GTL16 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 demonstrated dose-dependent inhibition of critical signal transduction events downstream of c-Met including Gab-1, Akt, Erk, PLC$_\lambda$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 sections taken at Day 16 of PF-04217903 treatment at 3mg, 10mg and 30 mg/kg/day in the GTL-16 model (Figure 2G), which correlated with the inhibition of c-Met phosphorylation (Figure 2E). Furthermore, Western blot analysis demonstrated a dose-dependent induction of activated caspase-3 levels at 3mg, 10mg and 30 mg/kg/day in the same GTL-16 study (Figure 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 tumor growth inhibition (TGI) was achieved in all five dose groups, with 10mg/kg and 30mg/kg dose levels demonstrating marked tumor size reduction of 68% and 84% respectively (Figure 3A). This dose-dependent TGI correlated with the decreased Ki67 expression in these tumors determined by IHC analysis (Fig. 3C). Phospho-RTK array analysis showed that besides c-Met, EGFR and PDGFRβ 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 three co-activated RTKs (c-Met, EGFR and PDGFR) as well as RTK downstream signaling. As shown in Figure 3B, 5 hours (Tmax) after oral dosing, PF-04217903 dose-dependently inhibited c-Met, Gab1, Erk1/2, and Akt phosphorylation, and induced apoptosis (cleaved caspase3) in U87MG xenograft tumors at all dose levels (5 mg/kg, 15 mg/kg and 50 mg/kg, PO/QD 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 PDGFRβ 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 to response of PF-4217903, we selected a panel of tumor cell lines that express high level of c-Met without MET gene amplification. SW480 and SW620 are two colon carcinoma cell lines derived from the same patient. SW480 was obtained from primary colon cancer tissue and SW620 was isolated one year later from a metastatic lymph node (42). FACS analysis of expression levels of c-Met and related RTKs demonstrated that SW480 expressed relatively low c-Met protein and high EGFR, but SW620 expressed high c-Met and low EGFR
PF-04217903 administered orally at 40 mg/kg/day, demonstrated 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 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 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 qPCR analysis (data not shown).

The HT-29 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-Δ160 and sf-Ron (43). PF-04217903, orally administered at 50 mg/kg/day, significantly inhibited c-Met phosphorylation (4F) and demonstrated a 40% decrease in HT29 tumor growth (Fig 4E). To investigate whether RON also contributes to progression of HT29 tumors, a RON shRNA was utilized 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 1) PF-04217903 mono-therapy increased phospho-AKT levels and did not induce tumor apoptosis, 2) combining PF-04217903 with RON shRNA knock down blocked phospho-AKT up-regulation observed in PF-04217903 mono therapy, 3) Ron shRNA knock down significantly increased activated caspase3 levels compared to the HT29 vector control tumors with or without PF-04217903 treatment (Fig 4E).

PF-04217903 also partially inhibited tumor growth of Colon205 human colon carcinoma, MDA-MB-231 human breast carcinoma and H292 human NSCLC xenograft tumors, that over-
express c-Met, by 44%, 43% and 39% respectively (Fig 4G-I). Collectively, these results indicate near complete inhibition of c-Met activity by PF-04217903 in the selected c-Met over-expressing models resulted in significant but incomplete tumor growth inhibition. PF-04217903 was well tolerated in mice. There was no animal body weight loss observed in any of the dose levels tested in the vivo studies.

**Anti-angiogenic 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 anti-angiogenic activity in vitro and in vivo.

PF-04217903 inhibited HGF stimulated c-Met phosphorylation in Human Umbilical Vascular Endothelial Cells (HUVEC) with an IC\textsubscript{50} value of 4.6 nM (Supplement Table 1). In endothelial cell functional assays, PF-04217903 inhibited HGF-mediated HUVEC survival (IC\textsubscript{50} = 12 nM), matrigel invasion (IC\textsubscript{50} = 27 nM) and induced HUVEC apoptosis (IC\textsubscript{50} = 7 nM) (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/day in U87MG tumors following 10-days of PF-04217903 treatment (Fig. 5A) indicating that inhibition of MVD correlated to significant antitumor efficacy (Fig 3A). c-Met and HGF have also been shown to regulate the secretion of pro-angiogenic 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-C), and decreased human VEGFA levels in the GTL-16 model (Fig 5C). This data suggests that the anti-angiogenic
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 1) harbor MET gene amplification, 2) exhibit a HGF/c-Met autocrine loop or 3) 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 pharmacological inhibition of this oncogene leads to tumor growth arrest and cell death (44). The robust anti-proliferation and anti-survival effects we observed upon inhibiting c-Met catalytic activity by PF-04217903 in MET amplified GTL-16 model is 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 demonstrate that near complete inhibition (>90%) of c-Met activity during
the entire dosing period is necessary to maximize therapeutic benefit. These pre-clinical findings provided the pharmacological 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 anti-proliferative 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 microvessal density 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 anti-angiogenic effect of PF-04217903 through direct inhibition of endothelial cell survival or indirect inhibition of angiogenic cytokine secretion by U87MG cells. Shojaei et al 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 extensively reported that these three 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 demonstrated 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 Gab1 activities only correlated with c-Met activity, but not with EGFR or PDGFRβ activities indicating a unique signaling cascade through the c-Met/Gab1 axis in the U87MG tumors. Emmanelle et al reported recently that increased c-Met and PDGFR expression level was observed in the glioblastoma patients that 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: 1) are these partial antitumor efficacies in c-Met overexpressing pre-clinical models significant enough to translate into clinical activity? 2) 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 oncogenic alterations, such as the KrasG12V mutation in SW620, the BrafV600E mutations in HT29 and colo-205, and the KrasG13D in the triple negative MDA-MB-231 breast carcinoma, indicating that c-Met overexpression and activation is 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 (c-Met monoclonal antibody) plus erlotinib demonstrated improved PFS and OS only in high c-Met expression NSCLC patients (3). Furthermore, RTK coactivation could also affect tumor response to targeted therapies (20,51).

Using phospho-RTK array analysis, we found multiple RTKs were concomitantly activated in these tumors such as EGFR, 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 co-activated 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 co-activated 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/sfRon were overexpressed and activated, we observed enhanced tumor growth inhibition by combining PF-04217903 with Ron shRNA knock down. 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 MAPK inhibition and upregulated AKT activity, suggesting 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 demonstrated a therapeutic benefit of the combination approach for c-Met inhibitors in c-Met overexpressing cancers. Since 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 compared with c-Met inhibitor single agent therapy in the majority of cancers that overexpress activated c-Met.

In summary, our studies demonstrate 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 co-driver in “oncogene co-activated” cancers.
References
51. Xu AM and Huang PH. Receptor Tyrosine Kinase Coactivation Networks in Cancer. Cancer Res 2010;70:3857-60.
**Table 1. Effect of PF-04217903 on c-Met-Dependent Cellular Phenotypes in Vitro**

<table>
<thead>
<tr>
<th>Assay</th>
<th>c-Met status</th>
<th>PF-04217903 Concentration IC₅₀ (nM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor Cell Phenotypes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation (MTT assay) of GTL-16 gastric carcinoma cells (mean IC₅₀)</td>
<td>Gene amplification</td>
<td>11.5</td>
</tr>
<tr>
<td>Proliferation (MTT assay) of NCI-H1993 lung carcinoma cells (mean IC₅₀)</td>
<td>Gene amplification</td>
<td>30</td>
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<td>Proliferation (MTT assay) of a panel of tumor cell linesᵇ (mean IC₅₀)</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 IC₅₀)</td>
<td>Gene amplification</td>
<td>31</td>
</tr>
<tr>
<td>HGF-stimulated HT29 cell Boyden Chamber Matrigel invasion (mean IC₅₀)</td>
<td>Overexpression</td>
<td>7</td>
</tr>
<tr>
<td>HGF-stimulated NCI-H441 cell migration (Acea System) (IC₅₀)</td>
<td>Overexpression</td>
<td>11</td>
</tr>
<tr>
<td>HGF-stimulated NCI-H441 cell invasion (Acea System) (IC₅₀)</td>
<td>Overexpression</td>
<td>12.5</td>
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<td><strong>Endothelial Cell Phenotypes</strong></td>
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<td>HGF-stimulated HUVEC survival (MTT assay) (mean IC₅₀)</td>
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<td>12.3</td>
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<tr>
<td>HGF-stimulated HUVEC Matrigel invasion (Acea System) (IC₅₀)</td>
<td>Normal</td>
<td>27</td>
</tr>
<tr>
<td>Apoptosis (ssDNA assay) of HUVEC cells (IC₅₀)</td>
<td>Normal</td>
<td>7.3</td>
</tr>
</tbody>
</table>

ᵃ IC₅₀ values were generated by curve fitting using a four-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 etc.

Definitions: NSCLC=non-small cell lung cancer; HUVEC=human umbilical vein endothelial cells.
Figure Legends:

Figure 1. Effect of PF-04217903 on (A) Met phosphorylation in GTL-16, NCI-H1993 and HT29 cells, (B) cell proliferation in GTL-16, NCI-H1993, U87MG, SW620 and HT29 cells, (C) GTL-16 cell apoptosis, and (D) NC1-H441 Cell matrigel invasion in vitro—(A) The cells were seeded in the 96-well plates and were treated with designated concentrations of PF-04217903 for one 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 access 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 utilizing the ssDNA Apoptosis ELISA Kit (Chemicon International). The ssDNA content of the cells was quantified as percent of control cells. (D) NC1-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 was assessed real time for 48 hours by the electronic sensor embedded in the bottom of the well using ACEA CIM Cell System. Invaded cells were also fixed and stained with Diff-Quick Fixative Solution and imaged by using the ImagePro imaging system.

Figure 2. Inhibition of c-Met Phosphorylation (A, D, E), Tumor Growth (B, C, 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 (250mm3—A, B, C, D, G, H) or (400mm3—E,F) were administered PF-04217903 orally or subcutaneous mini-pump infusion at the indicated dose or vehicle alone over the designated treatment schedule. An * denotes a significant difference from the control group (P < 0.05) as determined using one-way analysis of variance.

(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 OD treated / Mean OD untreated) X 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 c-Met/HGFR phosphorylation in tumors (% inhibition) and tumor growth inhibition (tumor growth rate was calculated as described by Jackson (52)) are expressed as Hill Function plots with variable Emax, slope and EC50 following 14-days subcutaneous pump infusion. (E) Inhibition of c-Met phosphorylation following 4-days of PF-04217903 treatment determined by IHC analysis. (F) Inhibition of c-Met and its downstream signaling pathways following 4-days of treatment determined by Western blot analysis. (G) Dose-dependent decrease in the number of Ki67 positive cells following 16-day drug treatment determined by IHC analysis. (H) induction of apoptosis (cleaved caspase3) following 16-days of PF-04217903 treatment in GTL-16 tumors determined by Western blot analysis.

**Figure 3. Inhibition of Tumor Growth (A), c-Met phosphorylation and downstream signal transduction (B), tumor cell proliferation (C) and survival (D) by PF-04217903 in U87MG Xenograft Model.** Athymic mice bearing established U87MG tumors (170mm3—A,C or 400mm3—B,D) were administered PF-04217903 orally at the indicated dose or vehicle alone over the designated treatment schedule. (A) Dose-dependent inhibition of U87MG tumor growth following 10-days of dosing. (B) Inhibition of c-Met phosphorylation and downstream signal transduction, and induction of apoptosis (cleaved caspase3) following 3-days of PF-04217903 treatment by Western blot analysis. (C) Dose-dependent decrease in the number of Ki67 positive cells following 4-day drug treatment by IHC analysis. (D) Levels of phospho and total EGFR or PDGFRβ levels in the U87MG xenograft tumors following 3-days of PF-04217903 treatment by Western blot analysis.

An * denotes a significant difference from the control group (P < 0.005) as determined using one-way analysis of variance.

**Figure 4. Antitumor efficacy of PF-04217903 in SW620 (B,C), HT29 (E,F), Colo205 (G), MDA-MB-231 (H) and H292 (I) xenograft tumor models.** Athymic mice bearing established tumors (~200 mm3) were administered PF-04217903 orally at the indicated dose or vehicle alone over the designated treatment schedule. (A) Protein expression level of c-Met, EGFR and related RTKs in SW480 and SW620 colon carcinoma cells determined by FACS analysis. (B) SW480 and SW620 tumor growth following 15 or 25 days of PF-04217903 treatment respectively. (C) Inhibition of c-Met phosphorylation and induction of c-Met protein
upregulation following 25-day of dosing in SW620 model. (D) Ron shRNA selectively knocked down Ron protein levels in HT29 cells determined by FACS analysis. (E) Inhibition of tumor growth in HT29 or HT29-shRon models. (F) Inhibition of c-Met/Ron mediated signal transduction and tumor cell survival (cleaved caspase3) in HT29 or HT29-shRon models determined by Western blot analysis. (G) Inhibition of tumor growth by PF-04217903 in Colo205 xenograft model. (H) Inhibition of tumor growth by PF-04217903 in MDA-MB-231 xenograft model. (I) Inhibition of tumor growth by PF-04217903 in H292 xenograft model. An * denotes a significant difference from the control group (P < 0.05) as determined using one-way analysis of variance.

Figure 5. Effect of PF-04217903 on Tumor Microvessel Density (A) or Secretion of Pro-angiogenic Factors (B, C) in Tumor Xenografts in Vivo. Athymic mice bearing established GTL-16 or U87MG xenografts were administered PF-04217903 orally at the indicated dose levels or vehicle. (A) Inhibition of microvessel density (CD-31) in U87MG tumors following 10-day of PF-04217903 treatment. (B) Inhibition of human IL-8 serum levels in U87MG tumor bearing mice following 10-days of PF-04217903 treatment. (C) Inhibition of human VEGFA and IL-8 serum levels in GTL-16 tumor bearing mice following 4-days of PF-04217903 treatment. An * denotes a significant difference vs. the control group (P < 0.05) and a # denotes a significant difference vs. the control group (P < 0.001) as determined using one-way analysis of variance.
Figure 1

A. 

B. 

C. 

D.
Figure 2

A. (Cleaved Caspase-3)

B. (Tumor Growth)

C. (Met Phosphorylation)

D. (Met Activity)

E. (Vehicle vs. 3mg/kg)

F. (PF-04217903 Dosage vs. Met Phosphorylation and Tumor Growth)

G. (Ki67 Positive Cells)

H. (GAPDH)

Met Activity:

IC$_{50}$ = 10 nM

IC$_{90}$ = 45 nM

R$^2$ = 0.91

TGI:

IC$_{50}$ = 13 nM

IC$_{90}$ = 80 nM

R$^2$ = 0.82
Figure 3

A.

![Graph showing tumor volume over study days for different dosages of PF-04217903.]

B.

![Blots for various proteins (Phospho-Met, Total-Met, Phospho-Gab1, Total Gab1, Phospho-Erk 1/2, Total-Erk 1/2, Phospho-Akt(S473), Total-Akt, cleaved Caspase3, GAPDH) at different dosages (0mg, 5mg, 15mg, 50mg).]

C.

![Bar graph showing percent Ki67 positive cells at different PF-04217903 dosages (0mg, 1.3mg, 4.0mg, 13.3mg, 40mg).]

D.

![Blots for various proteins (Phospho-EGFR, Total-EGFR, Phospho-PDGFRβ, Total-PDGFRβ, GAPDH) at different dosages (0mg, 5mg, 15mg, 50mg).]
Figure 5

A.

B.

C.
Molecular Cancer Therapeutics

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

Helen Y Zou, Qiuhua Li, Joseph Lee, et al.

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