An Orally Available Small-Molecule Inhibitor of c-Met, PF-2341066, Exhibits Cytoreductive Antitumor Efficacy through Antiproliferative and Antiangiogenic Mechanisms

Helen Y. Zou, Qiuhua Li, Joseph H. Lee, Maria E. Arango, Scott R. McDonnell, Shinji Yamazaki, Tatiana B. Koudriakova, Gordon Alton, Jingrong J. Cui, Pei-Pei Kung, Mitchell D. Nambu, Gerrit Los, Steven L. Bender, Barbara Mroczykowski, and James G. Christensen

Departments of Cancer Biology, Pharmacokinetics, Dynamics, and Metabolism, Biochemical Pharmacology, Medicinal Chemistry, Oncology, and Molecular Biology, Pfizer Global Research and Development, La Jolla Laboratories, La Jolla, California

Abstract

The c-Met receptor tyrosine kinase and its ligand, hepatocyte growth factor (HGF), have been implicated in the progression of several human cancers and are attractive therapeutic targets. PF-2341066 was identified as a potent, orally bioavailable, ATP-competitive small-molecule inhibitor of the catalytic activity of c-Met kinase. PF-2341066 was selective for c-Met (and anaplastic lymphoma kinase) compared with a panel of >120 diverse tyrosine and serine-threonine kinases. PF-2341066 potently inhibited c-Met phosphorylation and c-Met–dependent proliferation, migration, or invasion of human tumor cells in vitro (IC50 values, 5–20 nmol/L). In addition, PF-2341066 potently inhibited HGF-stimulated endothelial cell survival or invasion and serum-stimulated tubulogenesis in vitro, suggesting that this agent also exhibits antiangiogenic properties. PF-2341066 showed efficacy at well-tolerated doses, including marked cytoreductive antitumor activity, in several tumor models that expressed activated c-Met. The antitumor efficacy of PF-2341066 was dose dependent and showed a strong correlation to inhibition of c-Met phosphorylation in vivo. Near-maximal inhibition of c-Met activity for the full dosing interval was necessary to maximize the efficacy of PF-2341066. Additional mechanism-of-action studies showed dose-dependent inhibition of c-Met–dependent signal transduction, tumor cell proliferation (Ki67), induction of apoptosis (caspase-3), and reduction of microvessel density (CD31). These results indicated that the antitumor activity of PF-2341066 may be mediated by direct effects on tumor cell growth or survival as well as antiangiogenic mechanisms. Collectively, these results show the therapeutic potential of targeting c-Met with selective small-molecule inhibitors for the treatment of human cancers.

Introduction

An emerging paradigm in oncology is that robust clinical efficacy can be obtained with inhibitors directed toward oncogenic receptor tyrosine kinases (RTK) that are mutated or otherwise dysregulated in selected advanced tumors (1, 2). Recent examples of successful therapeutic intervention with RTK inhibitors include imatinib in gastrointestinal stromal tumors (GIST) with mutant c-Kit, erlotinib in non–small cell lung cancer (NSCLC) with mutant and/or amplified epidermal growth factor receptor, trastuzumab in breast cancers with amplified/elevated HER-2, and sunitinib targeting the von Hippel-Lindau (VHL)-dependent vascular endothelial growth factor (VEGF) pathway in renal cell carcinoma (RCC; ref. 3). In addition to the aforementioned RTKs, c-Met is one of most frequently genetically altered or otherwise dysregulated RTKs in advanced cancers, implicating it as an attractive therapeutic target (4).

c-Met is the prototypic member of a subfamily of RTKs, which also includes RON. c-Met is structurally distinct from other RTKs and is the only known high-affinity receptor for hepatocyte growth factor (HGF), also known as scatter factor (SF; refs. 5, 6). Binding of HGF to the c-Met extracellular domain results in receptor multimerization and phosphorylation of multiple tyrosine residues at the intracellular region (5, 6). Tyrosine phosphorylation at the c-Met juxtamembrane, catalytic, and cytoplasmic domains regulate the internalization, catalytic activity, and docking of regulatory substrates, respectively (6–9). Activation of c-Met results in the binding and phosphorylation of adaptor proteins, such as growth factor receptor binding protein 2 (Grb2)–associated binding protein 1 (Gab1), Grb2, Src homology and collagen (Shc), and c-Cbl and subsequent activation of signal transducers such as phosphoinositide-3-kinase, Akt, peritoneal lymphocyte-γ (PLC-γ), signal transducers and activators of transcription (STAT), and extracellular signal-regulated kinases 1 and 2 (ERK1 and 2; ref. 10).

c-Met and HGF are expressed in numerous tissues, and their expression is normally confined predominantly to cells of epithelial and mesenchymal origin, respectively (11, 12). c-Met and HGF have been shown to be important in epithelial-mesenchymal interaction and regulation of cell migration, invasion, cell proliferation and survival, angiogenesis, morphogenic differentiation, and organization of three-dimensional tubular structures (e.g., renal tubular cells) during mammalian development and tissue repair and homeostasis (10, 13–16).

Although the controlled regulation of c-Met and HGF have been shown to be important in mammalian development and tissue homeostasis, their dysregulation is implicated in metastatic cancer progression. c-Met and HGF are highly expressed relative to surrounding tissue in numerous cancers, and their expression correlates with poor patient prognosis (10). 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 (17–22). Furthermore, amplification of the c-Met gene locus was detected in patients with gastric, metastatic colorectal cancer, and esophageal adenocarcinoma (23, 24). Cell lines engineered to express high levels of c-Met and HGF (autocrine loop) or mutant c-Met displayed a proliferative, motogenic, and/or invasive phenotype and grew as metastatic tumors in nude mice (25–29). Furthermore, HGF and c-Met have been implicated in the regulation of tumor angiogenesis through the direct proangiogenic properties of HGF or through the regulation of secretion of angiogenic factors, including VEGFA, interleukin-8 (IL-8), and thrombospondin-1 (30–33).

Although a number of inhibitors of the c-Met pathway have been identified, including HGF kringle variants/NK4, decoy receptors, or HGF neutralizing antibodies, the therapeutic utility of these approaches still requires clinical validation (4). Previously described small-molecule inhibitors of c-Met (e.g., SU11274 or PHA-665752) have been limited to described small-molecule inhibitors of c-Met (e.g., SU11274 or PHA-665752) have been limited to described small-molecule inhibitors of c-Met (e.g., SU11274 or PHA-665752) have been limited to described small-molecule inhibitors of c-Met (e.g., SU11274 or PHA-665752) have been limited to described small-molecule inhibitors of c-Met (e.g., SU11274 or PHA-665752). The present studies describe the identification and characterization of PF-2341066, an orally available ATP-competitive and selective small-molecule inhibitor of c-Met. PF-2341066 potently inhibited c-Met phosphorylation and signal transduction, as well as c-Met-dependent oncogenic phenotypes of tumor cells and endothelial cells in vitro and showed antitumor efficacy in tumor models at well-tolerated doses in vivo. Antitumor mechanism-of-action studies indicated that the antitumor activity of PF-2341066 may be mediated by both direct effects on tumor cell growth or survival, as well as antiangiogenic mechanisms.

Materials and Methods

**Compound**

PF-2341066 [(R)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl)-1H-pyrrozol-4-yl]-pyridin-2-ylamine; Supplementary Fig. S1] was synthesized at Pfizer, La Jolla Laboratories.

**Biochemical Kinase Assays**

c-Met catalytic activity was quantitated 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 consumption was measured by a decrease in absorbance at 340 nm by spectrophotometry at designated time points. To determine $K_i$ values, PF-2341066 was introduced into test wells at various concentrations in the presence of assay reagents and incubated for 10 min at 37°C. The assay was initiated by the addition of the c-Met enzyme.

**Cells**

Unless otherwise mentioned, cells were acquired from the American Type Culture Collection. GTL-16 gastric carcinoma cells and T47D breast carcinoma cells engineered to express human wild-type c-Met or its mutant variant c-Met Y1235D were donated by Dr. Paolo Comoglio or Dr. Marialavora DiRenzo (both from the University Turino Medical School, Candiolo, Italy), respectively. All cells were cultured in recommended media and serum concentration, and unless otherwise indicated, cell culture reagents were obtained from Life Technologies, Inc. Cells were maintained at 37°C in a humidified atmosphere with 5% to 10% CO₂ and maintained using standard cell culture techniques.

**Antibodies and Growth Factors**

Anti–total human c-Met was acquired from Zymed/Invitrogen. Anti–CD31 and anti–phospho-tyrosine (PY-20) were acquired from Santa Cruz Biotechnology. Anti–mouse c-Met was acquired from R&D Systems. Anti–phospho–c-Met; anti–total and phospho-Gab1; anti–total and phospho-Akt; anti–total and phospho-ERK1 and 2; and STAT5 were acquired from Cell Signaling Technologies. Antibodies used in immunohistochemistry studies included anti–phospho–c-Met (Biosource International/Invitrogen) and anti–Ki67 (DakoCytomation). Growth factors used in cell assays were each acquired from R&D Systems.

**Cell Assays**

All experiments were done under standard conditions (37°C and 5% CO₂). IC₅₀ values were calculated by concentration-response curve fitting using a Microsoft Excel–based four-parameter method.

**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-2341066 for 1 h and/or appropriate ligands for the designated times, cells were washed once with HBSS supplemented with 1 mmol/L NaVO₃, and protein lysates were generated from cells. Subsequently, phosphorylation of selected protein kinases was assessed by a sandwich ELISA method using specific capture antibodies used to coat 96-well plates and a detection antibody specific for phosphorylated tyrosine residues. Antibody-coated plates were (a) incubated in the presence of protein lysates at 4°C overnight; (b) washed seven times in 1% Tween 20 in PBS; (c) incubated in a horseradish peroxidase–conjugated anti–total-phosphotyrosine (PY-20) antibody (1:500) for 30 min; (d) washed seven times again; (e) incubated in 3,3’,5,5’-tetramethyl benziendioxide peroxidase substrate (Bio-Rad) to initiate a colorimetric reaction that was stopped by adding 0.09 N H₂SO₄; and (f) measured for absorbance in 450 nm using a spectrophotometer.

**Cell proliferation/survival assays.** Tumor cells were seeded in 96-well plates at low density in media supplemented with 10% FBS (growth media) and transferred to serum-free media (0% FBS and 0.04% BSA) after 24 h. Appropriate controls or designated concentrations of PF-2341066 were added to each well, and cells were incubated for 24 to 72 h. Human umbilical vascular endothelial cells (HUVEC) (passage 3) were seeded in 96-well plates in EGM2 media for 5 to 6 h at ≥20,000 cells per well and transferred to serum-free media (Cell Applications) overnight. The following day, appropriate controls or designated concentrations of PF-2341066 were added to each well, and after 1 h incubation, HGF was added to designated wells at 100 ng/mL. A 5-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega) was done to determine the relative tumor cell or HUVEC numbers.

**Tumor cell migration and Matrigel invasion assays.** NCI-H441 cell migration and Matrigel invasion were determined using a modified commercially available kit (BD Biosciences). Briefly, cells in log growth phase were trypsinized and suspended in serum-free media (with 0.04% BSA) at a density of 400,000 cells/mL. Designated control or treated suspended cells (0.5 mL) were added to each migration or invasion chamber (i.e., plate inserts) and incubated at 37°C for 22 h. In addition, 25 ng/mL HGF (0.75 mL) was added to the lower well of each companion plate to attract cells from migration or invasion chamber plate inserts at the top of the companion plate. Cells that invaded or migrated to the lower wells were then fixed and stained with 4,6-diamidino-2-phenylindole for 15 min at 37°C and washed twice with TBS, and the number of invading cells was quantitated using Image-Pro Plus software over a composite of five microscopic images (Media Cybernetics).

**HUVEC Matrigel invasion assay.** A549 cells were seeded at 1–2 × 10⁵ cells/mL in Matrigel (BD Biosciences) diluted 1:80 in starvation media (Cell Applications), supplemented with HGF (100 ng/mL) and/or different concentrations of PF-2341066, was added (50 μL) to designated wells and allowed to solidify at 37°C for 2 h. HUVEC were subsequently collected in starvation media at 60,000 cells/mL and treated with 100 ng/mL HGF and/or designated concentrations of PF-2341066 for 30 min at 37°C, and 100 μL of suspended cells were transferred to the top of
the Matrigel layer in the coated ACEA plate. The ACEA plate was connected to the ACEA Device Station at 37°C, and HIV-1, which invaded through Matrigel were monitored in real time by an ACEA Sensor Analyzer for 24 h and quantified using ACEA RT-CE5 Integrated Software.

Madin-Darby canine kidney cell scattering assay. Madin-Darby canine kidney cell (MDCK) scattering assays were done as described previously (34).

Human dermal microvascular endothelial cells vascular sprouting assay. Approximately 500 human dermal microvascular endothelial cells (HMVEC) (Clonetics) were added to EGM-2 medium containing 0.2% methylcellulose and transferred to U-bottomed 96-well plates to form spheroids overnight. Spheroids were supplemented with 2 mg/mL fibronogen and 4% FBS with vehicle or designated concentrations of PF-2341066 in 48-well thrombin-coated plates and incubated in EGM-2 containing 4% FBS at 37°C and 5% CO2. Endothelial tube formation was assessed, and images were captured on day 7 with an inverted microscope and Olympus BX60 digital camera.

Apoptosis assay. GTL-16 cells were seeded in 96-well plates at 40,000 cells per well. Designated concentrations of PF-2341066 or vehicle were added to the wells in serum-free media, and cells were incubated in 37°C and 5% CO2 for 48 h. The ssDNA Apoptosis ELISA kit (Chemicon) was used to detect apoptosis.

In vivo Studies

Animals. Female or male nu/nu mice (5–8 weeks old) 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-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.

S.c. xenograft models in athymic mice. Cells were harvested, pelleted by centrifugation at 450 x g for 5 to 10 min, and resuspended in sterile serum-free medium supplemented with 30% to 50% Matrigel (BD Biosciences). Cells (2–5 x 106 in 100 μL) were implanted s.c. into the hind-flank region of each mouse and allowed to grow to the designated size before the administration of compound. For studies with NCI-H441, DLD-1, and MDA-MB-231 xenografts, MRC-5 cells were added to tumor cells at a 2:1 ratio (tumor cells/MRC-5) before implantation as a source of bioactive and MDA-MB-231 xenografts, MRC-5 cells were added to tumor cells at a 2:1 ratio (tumor cells/MRC-5) before implantation as a source of bioactive

Results

PF-2341066 potently inhibited the catalytic activity of c-Met and its oncogenic variants in vitro. PF-2341066 (Supplementary Fig. S1) was selected for further characterization based on screens designed to identify inhibitors of c-Met. PF-2341066 was shown to be a potent ATP-competitive inhibitor of recombinant, human c-Met kinase activity with a mean Ki of 4 nmol/L. In cell-based assays, PF-2341066 inhibited HGF-stimulated or constitutive total tyrosine phosphorylation of wild-type c-Met, with a mean IC50 value of 11 nmol/L across a panel of human tumor and endothelial cell lines (Supplementary Table S1). PF-2341066 showed similar potency against c-Met phosphorylation in mIMCD3 mouse (IC50, 5 nmol/L) or MDCK canine (IC50, 20 nmol/L) epithelial cells.

c-Met–activating mutations have been identified in several human cancers and provide a strong rationale for clinical proof of concept studies. To address differences in potency against mutant variants of c-Met, PF-2341066 was evaluated for its ability to inhibit c-Met phosphorylation across a panel of cell lines expressing c-Met mutations (Supplementary Table S1). In these studies, PF-2341066 exhibited improved or similar activity against NHEH3T3 cells engineered to express c-Met ATP-binding site mutants V1092I (IC50, 19 nmol/L) or H1094R (IC50, 2 nmol/L) or the P-loop mutant M1250T (IC50, 15 nmol/L) compared with NHEH3T3 cells expressing wild-type receptor (IC50, 13 nmol/L). In contrast, a marked shift in potency was observed against cells engineered to express c-Met activation loop mutants Y1230C (IC50, 127 nmol/L) and Y1235D (IC50, 92 nmol/L) compared with wild-type receptor. PF-2341066 also potently inhibited c-Met phosphorylation in NCI-H69 (IC50, 13 nmol/L) and HOP92 (IC50, 16 nmol/L) cells, which express the endogenous c-Met variants R988C and T1010I, respectively.

Kinase selectivity of PF-2341066. PF-2341066 was evaluated in >120 biochemical kinase assays done at Upstate, Inc., or Pfizer and was determined to be more than 100-fold selective for c-Met compared with the majority (>90%) of kinases evaluated (data not shown). In cell-based assays to follow up initial biochemical data, PF-2341066 was >1,000-fold selective for the VEGFR2 and PDGFR β RTKs, >250-fold selective for IR and Lck, and ∼ 40- to 60-fold selective for Tie2, TrkA, and TrkB, all compared with c-Met (Supplementary Table S1). PF-2341066 was 20- to 30-fold selective for RON and Axl RTKs (Supplementary Table S1), however; pharmacologically relevant inhibitory activity (proposed as IC90 for 24 h) is unlikely to be achieved at efficacious doses of PF-2341066 based on assessment of in vivo pharmacodynamics for other RTK targets (data not shown). In contrast, PF-2341066 showed a near-equivalent IC50 value (24 nmol/L) against the nucleophosmin (NPM)-anaplastic lymphoma kinase (ALK) oncogenic fusion variant of the ALK RTK expressed by the KARPAS299 human anaplastic large cell lymphoma (ALCL) cell line. These data indicate that the pharmacologic activity of PF-2341066 is likely mediated by the inhibition of c-Met and ALK RTKs and their oncogenic variants. The pharmacologic properties of PF-02341066 were determined using one-way ANOVA.
in models of NPM-ALK-positive lymphoma will be described elsewhere.7

**PF-2341066 inhibited c-Met-dependent neoplastic phenotypes of cancer cells and angiogenic phenotypes of endothelial cells in vitro.** Because c-Met is implicated in a variety of tumor cell and tumor endothelial cell functions, PF-2341066 was evaluated in a series of cell-based functional assays. PF-2341066 inhibited human GTL-16 gastric carcinoma cell growth (IC50, 9.7 nmol/L), induced apoptosis in GTL-16 cells (IC50, 8.4 nmol/L), inhibited HGF-stimulated human NCI-H441 lung carcinoma cell migration and invasion (Supplementary Fig. S2, IC50s of 11 and 6.1 nmol/L, respectively), and inhibited MDCK cell scattering (IC50, 16 nmol/L; Supplementary Table S2).

In studies done using HUVEC, PF-2341066 inhibited HGF-stimulated c-Met phosphorylation (IC50, 11 nmol/L), cell survival (IC50, 14 nmol/L), and Matrigel invasion (IC50, 35 nmol/L; Supplementary Table S2). In addition, PF-2341066 inhibited serum-stimulated HMVEC branching tubulogenesis (formation of vascular tubes) in fibrin gels (Fig. 1). The strong correlation of IC50 values for inhibition of c-Met phosphorylation and c-Met-dependent phenotypes suggests that PF-2341066 pharmacologic activity in these assays is mediated by the inhibition of c-Met.

**Dose-dependent relationship of c-Met phosphorylation and tumor growth inhibition by PF-2341066 in c-Met-dependent tumor xenograft models.** Due to the robust expression of constitutively active c-Met and dependence of c-Met for tumor cell growth and survival, GTL-16 tumors were used to evaluate the relationship of c-Met target inhibition to tumor growth inhibition by PF-2341066 in vivo. To evaluate c-Met inhibition by PF-2341066, GTL-16 tumors were harvested at several time points following p.o. administration of PF-2341066 over a range of doses, and c-Met phosphorylation in tumors was quantitated by ELISA. In these studies, PF-2341066 showed the following as shown in Fig. 2 (A and B):

- At 50 mg/kg/day, 100% tumor growth inhibition correlated with complete inhibition of c-Met phosphorylation in GTL-16 tumors sustained for 24 h. At dose levels of >50 mg/kg/day, no further improvement of tumor growth inhibition was observed (data not shown).
- At 12.5 mg/kg/day, 60% tumor growth inhibition correlated with 80% to 90% inhibition of c-Met phosphorylation at 1 to 8 h, which decreased to 50% to 60% inhibition by 16 to 24 h.
- At 6.25 mg/kg/day, nonsignificant trend toward tumor growth inhibition correlated with 30% to 50% inhibition of c-Met phosphorylation at 1 to 8 h with full recovery by 16 h.

The following conclusions were apparent from these analyses: (a) complete inhibition of c-Met activity for 24 h is consistent with complete inhibition of tumor growth [50 mg/kg; 100% tumor growth inhibition (TGI)]; (b) potent inhibition of c-Met activity for only a portion of the schedule is consistent with suboptimal efficacy (12.5 mg/kg; 60% TGI); (c) inability to achieve >50% inhibition of c-Met activity (3.125, 6.25 mg/kg) is consistent with lack of significant TGI (Fig. 24 and B). In addition, a similar dose-dependent effect of PF-2341066 on tumor growth and c-Met phosphorylation (at 4 h) was observed using the U87MG glioblastoma xenograft model (Fig. 2C and D). Collectively, these studies suggest that near-complete inhibition of c-Met phosphorylation (>90% inhibition) for the duration of the administration schedule is necessary to maximize therapeutic benefit.

**Antitumor efficacy of PF-2341066 in c-Met-dependent human xenograft models.** Additional studies were done at optimal dose levels of PF-2341066 to assess its efficacy in human tumor xenograft models representative of cancer indications in which dysregulation of c-Met is implicated. Due to the lack of paracrine activation of human c-Met expressed in tumors by mouse HGF expressed by mouse mesenchymal cells, human xenograft models exhibiting constitutive c-Met activity were used as follows: (a) the GTL-16 human gastric carcinoma or Caki-1 RCC models that expresses elevated levels of constitutively active c-Met; (b) the U87MG human glioblastoma or PC-3 human prostate carcinoma model that expresses both HGF and c-Met comprising an autocrine loop; or (c) coimplantation of human tumor cells

---

In the GTL-16 model, PF-2341066 also showed near-complete inhibition of the growth of established tumors at 50 mg/kg/day in the U87MG glioblastoma model (Supplementary Fig. S3). Similar to the NGFR mechanism of action in c-Met–dependent tumor models, PF-2341066 was also evaluated for its effect on tumor mitotic index (Ki67) and apoptosis (activated caspase-3) using immunohistochemical (IHC) methods. A significant 4-fold decrease in Ki67 levels by study day 4 was observed at 25 and 50 mg/kg/day PF-2341066 in the GTL-16 (Fig. 4A–C) and U87MG (data not shown) models indicating a correlation with maximum antitumor efficacy. In addition, the evaluation of the 50 mg/kg dose over a time course indicated that the inhibition of mitogenesis was markedly more potent at days 3 and 4, suggesting that extended target inhibition maximizes this potentially cell cycle–dependent effect (Fig. 4D). Furthermore, a qualitative induction of activated caspase-3 at 25 and 50 mg/kg/day was observed in the GTL-16 model and at 50 mg/kg/day in the U87MG model (Supplementary Fig. S3). Similar to the modulation of Ki67, the increased apoptosis was dose dependent and correlated with the degree of antitumor efficacy of PF-2341066 (data not shown).

To evaluate the potential antiangiogenic mechanism of this compound in vivo, PF-2341066 was evaluated for time- and dose-dependent modulation of microvessel density (MVD) assessed by immunostaining for CD31 (platelet endothelial cell adhesion

![Image](https://example.com/image.png)
A significant dose-dependent reduction of CD31-positive endothelial cells was observed at 12.5, 25, and 50 mg/kg/day in GTL-16 tumors, indicating that inhibition of MVD showed a dose-dependent correlation to antitumor efficacy (Fig. 5A and B). At the 50 mg/kg/day dose level, PF-02340166 showed a significant 70% reduction of CD31-positive microvessels at study day 11 (Fig. 5A and B). In contrast, the effect on MVD was less apparent in the short-term time course study, suggesting that reduction of MVD requires longer term administration. In contrast to the GTL-16 model, only slight effects on MVD were observed in the U87MG model, suggesting that this potential antiangiogenic mechanism depends on tumor type.

C-Met and HGF have also been shown to regulate the secretion of proangiogenic factors, including VEGFA and IL-8 by tumor cells (32, 33). Therefore, the effect of PF-2341066 on VEGFA and IL-8 plasma levels was assessed to elucidate whether the effects on MVD observed following the administration of PF-2341066 were due to direct or indirect effects on tumor vessels. In these studies, PF-2341066 showed a significant dose-dependent reduction of human VEGFA and IL-8 plasma levels in both the GTL-16 and U87MG models (U87MG, Fig. 5C). Human VEGFA and IL-8 were not present in the plasma of non–tumor-bearing mice, indicating that the source of these plasma proteins was the tumor tissue (data not shown). A significant acute reduction of IL-8 or VEGFA plasma levels was observed after 1 or 2 days of PF-2341066 administration, respectively, in mice with size-matched tumor xenografts, indicating that observed effects were not due to differences in tumor burden (Fig. 5D).

**Effect of PF-2341066 on signal transduction pathways in tumors in vivo.** To investigate the effect of PF-2341066 on critical c-Met–dependent signal transduction events, immunoblot analysis of phosphorylation of molecules that regulate c-Met signal transduction was done using tumor tissue. In these studies, marked inhibition of phosphorylated c-Met, Akt, Erk, PLCγ1, and STAT5 levels was observed in GTL-16 tumors following p.o. administration of PF-2341066 (Fig. 6). Inhibition of these signaling events was dose dependent and was observed at pharmacologically relevant PF-2341066 dose levels, which correlated with the inhibition of c-Met phosphorylation, antitumor efficacy, and other mechanistic end points in vivo (Fig. 6).

**Discussion**

In the present studies, we describe a novel orally available inhibitor of c-Met and the consequences of inhibiting this RTK target in a variety of functional assays and tumor models. This prototype small molecule, PF-2341066, was identified as a potent ATP-competitive inhibitor of c-Met kinase, which inhibited c-Met phosphorylation across a panel of cell lines. The similar inhibitory activity against c-Met human and mouse epithelial cells has implications for interpreting potential antiangiogenic activity and safety profile of PF-2341066 during studies in tumor xenograft models in mice.

The clinical success with RTK inhibitors such as imatinib and erlotinib in patient populations exhibiting mutated RTK targets indicates that treatment of neoplasms with activating Met mutations comprises a viable clinical strategy for PF-2341066. PF-2341066 inhibited a variety of diverse mutant variants of c-Met in cellular assays, including those located at the ATP binding pocket (V1092I and H1094R), P-loop (M1250T), and juxtamembrane domain (R988C and T1010I). In contrast, PF-2341066 was less potent against the Y1230C and Y1235D mutant variants of c-Met located near the kinase domain activation loop. These data indicate that PF-2341066 activity is dependent on the location of
the mutation in the active site, which has unique implications in molecular modeling of c-Met inhibitory activity. These data indicate that selected mutations identified in papillary renal carcinoma, head and neck, and lung cancers are effectively inhibited by PF-2341066, and that particular mutations (e.g., activation loop) should be taken into account during patient selection (17, 19, 20, 22).

Evaluation of PF-2341066 across a structurally diverse cross-section of nearly 25% of all known tyrosine and serine-threonine kinases (35) indicated that it exhibited a high degree of selectivity at pharmacologically relevant concentrations. The exception to this was ALK, which was inhibited at pharmacologically relevant (24 nmol/L) concentrations by PF-2341066 in lymphoma cell lines expressing the NPM-ALK oncogenic fusion protein. Similar to BCR-Abl in chronic myelogenous leukemia, available literature indicates that ALK fusion proteins are a key disease driver of ALCL (36). In the present studies, disease models selected for study of c-Met–dependent pharmacology generally do not express ALK, suggesting that the pharmacologic activity of PF-2341066 is likely due to the inhibition of c-Met. However, due to the expression of wild-type ALK in selected endothelial cell populations, the relevance of ALK in mediating the pharmacologic activity of PF-2341066 cannot be discounted (37, 38).

The identification of PF-2341066 as a potent and selective inhibitor of c-Met provided some unique opportunities to investigate the pharmacologic consequences of c-Met inhibition. With the strong correlation between IC₅₀ values required for inhibition of c-Met phosphorylation and tumor or endothelial cell phenotypes, the present studies suggest that the pharmacologic effects of PF-2341066 are due to the inhibition of c-Met. These collective phenotypic data indicate that potential anticancer effects of PF-2341066 may range from (a) direct effects on tumor cell growth and survival to (b) antimitastatic properties through inhibition of tumor cell migration and invasion and may include (c) potential antangiogenic activity mediated through direct effects on the ability of endothelial cells to form functional vasculature. In addition, the strong correlation of inhibition of c-Met and c-Met–dependent phenotypes by PF-2341066 indicates that c-Met phosphorylation should function as a useful pharmacodynamic biomarker of inhibition of c-Met–mediated function in tumors. To this effect, studies relating pharmacodynamics to efficacy indicated near-complete inhibition of c-Met phosphorylation (>90% inhibition) for the duration of the administration schedule is necessary to maximize antitumor efficacy of PF-2341066. These data indicate that maintaining a sufficient degree of c-Met inhibition in patients will be critical in maximizing the therapeutic potential of this agent, and that a strategy to measure c-Met phosphorylation in patient tumor biopsies or surrogate tissue is warranted.

Following the identification of optimal dose levels of PF-2341066, studies were initiated to determine the sensitivity a variety of tumor models to this agent. The models selected for these studies represented different mechanisms by which c-Met has been reported to be activated in human tumors, including (a) paracrine activation by HGF expressed in tumor-associated mesenchymal tissue, (b) autocrine expression of both c-Met and HGF by tumor cells, and (c) expression of constitutively active c-Met due to increased expression or amplification of its gene locus. In these studies, PF-2341066 showed cytoreductive antitumor activity in models expressing high levels of constitutively activated c-Met, including GTL-16 gastric or Caki-1 renal carcinoma. These data indicate that gastric carcinoma patients with amplified c-Met or
renal patients with high levels of constitutively activated c-Met may comprise clinical populations that exhibit higher probability of objective response to PF-2341066. This concept is further supported by chemosensitivity of a panel of gastric cancer cell lines exhibiting amplified c-Met to a putative selective inhibitor of c-Met (39) and by increased expression and activation of c-Met in renal carcinoma cells devoid of functional VHL (40). Similarly, U87MG glioblastoma or PC-3 prostate carcinoma models in which tumor cells express both HGF and c-Met each exhibited a robust response to PF-2341066, supporting the concept that tumor types that exhibit autocrine production of c-Met and HGF may also be candidates for clinical studies. In contrast, although a robust response of the NCI-H441 NSCLC paracrine model to PF-2341066 was observed, other paracrine c-Met–expressing models such as MDA-MB-231 breast and DLD-1 colon carcinoma did not respond. These data suggest that response of populations in which wild-type c-Met is expressed in the absence of the constitutively active RTK or autocrine HGF may exhibit a heterogeneous response to c-Met–directed therapy. These data indicate that further study of molecular determinants sensitivities to c-Met–directed therapy is warranted. Chemosensitivity studies with selective c-Met inhibitors across a panel of human tumor cell lines expressing wild-type c-Met indicate that the response to these agents is heterogeneous and associated with selected molecular characteristics (4).

In addition to understanding molecular determinants of chemosensitivity, a basic understanding of the mechanism of action of PF-2341066 antitumor activity should also support a robust clinical strategy. As described above, PF-2341066 showed a direct effect on tumor cell growth and survival end points in vitro and dose-dependent inhibition of cell proliferation and induction apoptosis in tumors in vivo. Therefore, one mechanism of PF-2341066 is likely mediated via direct effects on tumor cell mitogenesis and apoptosis in tumor types in which dysregulation of c-Met is implicated in altered tumor cell growth regulation. In addition, Ki67 and activated caspase-3 were modulated in models that responded to PF-2341066 but were unaffected in those that did not (data not shown), indicating that these end points may represent markers of antitumor efficacy.

In the present studies, PF-2341066 also showed the ability to inhibit HGF-stimulated endothelial cell survival and migration, to inhibit endothelial cell tubulogenesis, and to reduce MVD in the GTL-16 model. In addition to their reported direct role in regulating endothelial cell function, c-Met and HGF are also implicated in the regulation of secretion of angiogenic factors by...
epithelial and tumor cells. In the present studies, PF-2341066 showed a robust modulation of VEGF and IL-8 in the GTL-16 and U87MG tumor models that correlated with its antitumor activity. Together, these data suggest that antiangiogenic activity observed with PF-2341066 may be mediated by direct or indirect mechanisms. If the antiangiogenic activity of PF-2341066 is dependent on the functional expression of c-Met by tumor cells, this will have implications in terms of the breadth of spectrum of antiangiogenic activity across tumor types. The absence of evidence of PF-2341066 antiangiogenic activity in the U87MG model and the absence of efficacy in the MDA-MB-231 and DLD-1 models suggest that the antiangiogenic activity of this agent is dependent on factors that are not common to all tumor types. Therefore, although PF-2341066 showed antiangiogenic properties in selected assays and models, it is not evident that this agent will exhibit broad spectrum antitumor activity through an antiangiogenic activity similar to VEGF pathway antagonists (41).

In summary, these studies illustrate the pharmacologic effects of PF-2341066 on a variety of pro-oncogenic tumor cell phenotypes, tumor models, and molecular mediators of cancer progression. The broad antitumor efficacy and good safety profile of this molecule suggest its potential as a novel agent for treatment of a variety of cancers. The variety of different responses to PF-2341066 observed in tumor cells and endothelial cells illustrates that this compound is likely to exert its antitumor effects through multiple distinct mechanisms. In addition, collective results indicate that the role of c-Met in the progression of cancers may differ depending on the cell lineage of origin and genetic context of a given tumor cell. The diverse role of c-Met in different tumor types as a regulator of tumor growth, survival, or metastasis will be a critical issue in the clinical development of c-Met inhibitors, monitoring of patient response, and design of clinical studies.

Acknowledgments

Received 12/4/2006; revised 1/27/2007; accepted 2/15/2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Paolo Comoglio and Dr. Maria Flavia DiRenzo for the original supply of GTL-16 cells and of T47D cells (expressing c-Met Y1235D or wild type), respectively.

References

An Orally Available Small-Molecule Inhibitor of c-Met, PF-2341066, Exhibits Cytoreductive Antitumor Efficacy through Antiproliferative and Antiangiogenic Mechanisms

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