A Novel Inhibitor of c-Met and VEGF Receptor Tyrosine Kinases with a Broad Spectrum of In Vivo Antitumor Activities

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Abstract

The c-Met receptor tyrosine kinase and its ligand, hepatocyte growth factor (HGF), are dysregulated in a wide variety of human cancers and are linked with tumorogenesis and metastatic progression. VEGF also plays a key role in tumor angiogenesis and progression by stimulating the proangiogenic signaling of endothelial cells via activation of VEGF receptor tyrosine kinases (VEGFR). Therefore, inhibiting both HGF/c-Met and VEGF/VEGFR signaling may provide a novel therapeutic approach for treating patients with a broad spectrum of tumors. Toward this goal, we generated and characterized T-1840383, a small-molecule kinase inhibitor that targets both c-Met and VEGFRs. T-1840383 inhibited HGF-induced c-Met phosphorylation and VEGF-induced VEGFR-2 phosphorylation in cancer epithelial cells and vascular endothelial cells, respectively. It also inhibited constitutively activated c-Met phosphorylation in c-met-amplified cancer cells, leading to suppression of cell proliferation. In addition, T-1840383 potently blocked VEGF-dependent proliferation and capillary tube formation of endothelial cells. Following oral administration, T-1840383 showed potent antitumor efficacy in a wide variety of human tumor xenograft mouse models, along with reduction of c-Met phosphorylation levels and microvessel density within tumor xenografts. These results suggest that the efficacy of T-1840383 is produced by direct effects on tumor cell growth and by an antiangiogenic mechanism. Furthermore, T-1840383 showed profound antitumor activity in a gastric tumor peritoneal dissemination model. Collectively, our findings indicate the therapeutic potential of targeting both c-Met and VEGFRs simultaneously with a single small-molecule inhibitor for the treatment of human cancers. Mol Cancer Ther; 12(6); 913–24. ©2013 AACR.

Introduction

The c-Met receptor tyrosine kinase (RTK) is the receptor for hepatocyte growth factor/scatter factor (HGF; reviewed in ref. 1). Binding of HGF to c-Met induces c-Met dimerization and phosphorylation of multiple tyrosine residues in the intracellular region, leading to activation of c-Met signaling pathways. c-Met signaling plays important roles in cell growth, survival, motility, and morphogenesis during embryonic development as well as in cancer cell proliferation, invasion, metastasis, tumor angiogenesis, and drug resistance (reviewed in refs. 1 and 2). Aberrant c-Met signaling, resulting from c-met genomic amplification, c-Met or HGF overexpression, or c-Met mutations, has been observed in a variety of human cancers (1, 2). Clinically, dysregulation of c-Met is correlated with a poor prognostic outcome (1, 2). Recently, constitutive activation of c-Met due to c-met amplification was also found to be a driver of cell proliferation and survival in several gastric cancer cell lines (3, 4) and has additionally been linked to acquired resistance to EGF inhibitors in lung cancers (5, 6). Thus, c-Met has been considered an appealing molecular target for cancer therapy (2, 7–9).

The receptors for VEGF, VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2), are members of the RTK family; they are mostly located in endothelial cells (reviewed in ref. 10). VEGF activation occurs through VEGF binding, which triggers receptor dimerization, tyrosine kinase activation, and phosphorylation of tyrosine residues. Of the 2 VEGFRs, VEGFR-2 is believed to be a major driver of tumor angiogenesis, which plays important roles in tumor malignancy (such as sustaining tumor growth) and in blood-borne metastasis (11). VEGF expression is upregulated by changes associated with cancer, including hypoxia, proto-oncogene activation, loss of tumor suppressor gene expression, and growth factor stimuli in tumors (12, 13). Its overexpression has been reported to correlate with the degree of vascularity, aggressive disease, and poor prognosis in the majority of human solid tumors (14–17), making the VEGF/VEGFR...
axis an attractive molecular target for cancer therapy. Indeed, the monoclonal anti-VEGF antibody bevacizumab (18) and several RTK inhibitors targeting VEGFRs, such as sunitinib (19), sorafenib (20), and pazopanib (21), have proven to be efficacious in clinical settings.

The above-mentioned findings highlight the possibility that simultaneous inhibition of c-Met and VEGFRs by a small-molecule inhibitor may result in broader and more potent antitumor efficacy. Here, we report the characterization of T-1840383, a novel ATP-competitive multitargeted kinase inhibitor that preferentially inhibits c-Met and VEGFR-2, in various functional cellular assays and the evaluation of its in vivo antitumor activities in various tumor xenograft models.

Materials and Methods

T-1840383

N-[4-[(2-[(cyclopropylcarbonyl)amino]imidazo[1,2-a]pyridin-6-yl]oxy)-3-fluorophenyl]-6-methyl-2-oxo-1-phenyl-2,3-dihydropyridine-3-carboxamide hydrochloride (T-1840383, Fig. 1A) was synthesized at Takeda Pharmaceutical Company, Ltd. For in vitro studies, T-1840383 was dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium immediately before use. For in vivo studies, T-1840383 was suspended in vehicle (0.5% methyl cellulose in distilled water), and the suspension was administered to animals within a week of preparation.

Kinase inhibition assays

Kinase inhibition of c-Met, VEGFRs, Tie-2, and Flt-3 was investigated using AlphaScreen tyrosine kinase technology (PerkinElmer). To evaluate kinase selectivity, a single concentration (1 μmol/L) of T-1840383 was tested using 256 kinases by KinaseProfiler (Millipore), and then IC_{50} values for the selected 66 kinases were determined using KinaseProfiler IC_{50} Express (Millipore).

Cell lines

Unless otherwise mentioned, cells were obtained from the American Type Culture Collection. Five gastric cancer cell lines (GSU, Kato III, KE-97, MKN45, and NUGC-4) were obtained from the RIKEN BioResource Center and 4 gastric cancer cell lines (SNU-484, -620, -638, and -668) were obtained from the Korean Cell Line Bank. EBC-1 squamous cell lung cancer cells and SUIT-2 pancreatic cancer cells were obtained from the Health Science Research Resources Bank. OE33 esophageal adenocarcinoma cells were obtained from the European Collection of Animal Cell Cultures. Human umbilical vein endothelial cells (HUVEC) and normal human dermal fibroblasts (NHDF) were purchased from Cambrex. All cells were cultured in recommended media and serum concentration, unless otherwise indicated. No authentication was done by the authors.

Tube formation assay

Human umbilical vein endothelial cells (1,000 cells/well) and NHDFs (10,000 cells/well) were cocultured in 96-well plates with 60 ng/mL VEGF (R&D Systems) and designated concentrations of T-1840383 for 7 days. After fixation with 70% ethanol, the cells were incubated with anti-human CD31 monoclonal antibody (R&D Systems) followed by Alexa Fluor 488-conjugated antimouse IgG polyclonal antibody (Invitrogen). Tube formation by endothelial cells was visualized using fluorescence imaging (Discovery-1 system, Molecular Devices) according to the manufacturer’s protocol, “Angiogenesis Tube Formation.” The filament length and number of branch points were quantified by using Metamorph software (Molecular Devices).

Cell scattering assay and E-cadherin expression analysis

A549 cells were seeded onto poly lysine-coated glass-bottom dishes (2,000 cells/dish) and were allowed to form colonies of appropriate size for 5 days. Controls or T-1840383 were added to each dish. After 1-hour incubation, 50 ng/mL HGF (R&D Systems) was added to the dishes. Cell scattering was stimulated for 48 hours under a mild hypoxic condition (3% O_2). Cells were stained using a Diff-Quik stain kit (Sysmex). For E-cadherin expression analysis, scattering-stimulated cells were fixed with 4% paraformaldehyde and were subjected to immunofluorescence staining using anti-E-cadherin primary antibody and Alexa Fluor 488-conjugated antirabbit IgG antibody. Nucleic acid was stained with propidium iodide.

Matrigel invasion assay

Cell invasion was assessed using Matrigel Invasion Chambers (BD Biosciences). A549 cells or SUIT-2 cells were placed in the top chamber, and T-1840383 was added to both the top and bottom chambers. Cell invasion was then initiated by adding HGF to the bottom chamber (50 ng/mL). After 72 hours, noninvading cells were removed with cotton swabs. Cells that invaded through the Matrigel were stained using a Diff-Quik stain kit, and the number of invading cells was quantified using WinROOF software (Mitani Corp.).

Cell proliferation assay

Cancer cells and HUVECs were seeded in 96-well plates in appropriate media with 10% FBS and human endothelial serum-free medium (Invitrogen), respectively. The following day, serial dilutions of T-1840383 or 0.1% DMSO were added to each well. Cancer cells were then incubated for a further 72 hours. Proliferation of HUVECs was stimulated with 60 ng/mL recombinant human VEGF for 120 hours. After incubation, cell proliferation was determined using a Cell Counting Kit-8 (DOJINDO Laboratories). IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism (GraphPad Software, Inc.).

Western blotting

Antibodies were purchased from Cell Signaling Technology. For analysis of A549 cells and HUVECs, c-Met
and VEGFR2 phosphorylation were stimulated with 50 ng/mL HGF and 100 ng/mL VEGF for 10 and 5 minutes, respectively. All cells were lysed in RIPA buffer (Pierce) with protease and phosphatase inhibitors. In the case of the tumor samples, tissues were homogenized in RIPA buffer with protease and phosphatase inhibitors. Samples were then subjected to Western blot analysis. Detection was carried out using enhanced chemiluminescence reagent (GE Healthcare).

Tumor xenograft models
All animal experiments were conducted according to the guidelines of the Takeda Experimental Animal Care and Use Committee. Athymic nude mice (BALB/cA Jcl-nu/nu) were purchased from Japan CLEA. For tumor models, cancer cells in Hanks balanced salt solution (HBSS; Gibco, Invitrogen Corp.) were subcutaneously inoculated into the hind flank of 6- to 7-week-old mice (day 0). For the study with MKN45, cells were suspended in a 1:1 mixture of Matrigel and HBSS and then inoculated. After the tumors were established, mice bearing tumors of appropriate size were randomized. Once-daily oral administration of T-1840383 or vehicle was initiated on the next day after randomization. Tumor volume was measured twice weekly with Vernier calipers and calculated as \( \text{volume} = \frac{\text{length} \times \text{width}^2}{2} \). Percentage treated/control ratio (T/C%) was calculated by dividing the change in volume of the tumors in the treated mice by the change in volume in the mice treated by vehicle, and statistical analysis was conducted as described previously (22).

For a peritoneal dissemination model, NUGC-4 cells expressing luciferase (NUGC-4-luc cells) were generated by stable transfection and inoculated via intraperitoneal injection into nude mice (1 \( \times \) 10\(^6\) cells). Daily oral administration (5 days per week) of T-1840383 at 5 mg/kg or vehicle was initiated 2 weeks after inoculation. Tumor growth was monitored on the basis of determination of
emitted bioluminescence (photons/s) 10 minutes after intraperitoneal administration of d-luciferin (1.5 mg/kg). Mice were euthanized when they became moribund. Statistical analysis was conducted by a log-rank test using GraphPad Prism.

**Immunohistochemistry**

Mice (BALB/c A/Jcl-nu/nu) bearing A549 tumors were treated with T-1840383 at the indicated doses for 3 days. The dissected tumors were fixed in paraformaldehyde and then paraffin embedded. For visualization of the vessels, immunohistochemical staining for CD31 was conducted using rabbit anti-CD31 polyclonal antibody (Spring Bioscience) and the immunoperoxidase technique with diaminobenzidine tetrahydrochloride as the chromagen. Ki67 staining for proliferating cells and TUNEL for apoptotic cells was conducted using anti-Ki67 antibody (Dako) and ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore), respectively. Stained sections were scanned with a NanoZoomer Digital Pathology system (Hamamatsu Photonics). Image processing was conducted by analyzing 4 or 5 randomly selected fields of each tumor using WinROOF software (Mitani Corporation).

**Results**

**T-1840383 is a potent kinase inhibitor that targets both c-Met and VEGF receptors**

The selectivity of T-1840383 (Fig. 1A) was profiled against a panel of protein kinases and lipid kinases (Table 1). T-1840383 inhibited c-Met tyrosine kinase with an IC\textsubscript{50} value of 1.9 nmol/L. It also inhibited VEGFR tyrosine kinases with IC\textsubscript{50} values of 7.7, 2.2, and 5.5 nmol/L for VEGFR-1, -2, and -3, respectively. The structure of T-1840383 has little in common with other VEGFR or c-Met selective inhibitors, except for TAK-593 (Supplementary Fig. S1). T-1840383 was found to inhibit several kinases including c-Mer, Ret, Ron, Rse, Tie-2, and TrkA as well as mutants of Abi, c-Kit, fibroblast growth factor receptors \textit{a} (FGFR\textit{a}) and platelet-derived growth factor receptors \textit{a} (PDGF\textit{a}) with similar IC\textsubscript{50} values to those for c-Met and VEGFRs.

**T-1840383 inhibits HGF-induced c-Met phosphorylation and HGF-dependent cellular phenotypes**

In cell-based assays using A549 cells, T-1840383 inhibited HGF-stimulated c-Met phosphorylation with an IC\textsubscript{50} value of 8.8 nmol/L (Fig. 1B). It also inhibited A549 cell

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**Table 1. Inhibitory activities of T-1840383 against 72 kinases**

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\textsuperscript{a}For these 6 kinases, the IC\textsubscript{50} values were determined by the AlphaScreen assay. For others, the IC\textsubscript{50} values were determined using KinaseProfiler IC\textsubscript{50} Express (Millipore).
scattering stimulated with HGF (Fig. 1C). Because HGF signaling has been reported to trigger destabilization of adherens junctions by downregulation of E-cadherin (23), expression level of E-cadherin was examined in a similar cell-scattering assay. As expected, HGF-stimulated cell scattering was accompanied by diminished expression of E-cadherin on cell membrane, which was inhibited by T-1840383 in a concentration-dependent manner (Fig. 1D). T-1840383 also displayed similar potency against HGF-induced A549 cell invasion in a Matrigel invasion assay (Fig. 1E and Supplementary Fig. S2).

**T-1840383 inhibits c-Met–dependent cell proliferation in vitro**

The effects of T-1840383 on cell proliferation were evaluated using a panel of cancer cell lines and HUVECs. The antiproliferative potency varied widely, with the IC_{50} values ranging from 1.8 nmol/L to >10,000 nmol/L (Fig. 1F). With the exception of HUVECs, all T-1840383–sensitive cell lines (IC_{50} <25 nmol/L; SNU-5, SNU-638, MKN45, NUGC-4, and Hs746T gastric cancer cells as well as EBC-1 non–small cell lung carcinoma cells) were shown to overexpress constitutively activated c-Met protein (Supplementary Fig. S3A). These cell lines are also known to harbor *met* genomic amplification (3, 4, 24, 25), suggesting that constitutive activation of c-Met is dependent on genomic amplification. In MKN45 cells, T-1840383 inhibited activation of c-Met and the downstream effectors ERK1/2 and AKT in a concentration-dependent manner (Supplementary Fig. S3B). In contrast, OE33 esophageal adenocarcinoma cells and KATOIII and SNU-620 gastric cancer cells were much less sensitive to T-1840383 despite the fact that c-Met was constitutively active in those cell lines. Other insensitive cell lines (IC_{50} >10,000 nmol/L) did not show constitutively activated c-Met. These results suggest that constitutively active c-Met is a necessary but not sufficient predictor of *in vitro* cancer cell response to T-1840383.

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**Figure 2.** T-1840383 inhibits VEGFR-2 phosphorylation and VEGF signaling in HUVECs. A, HUVECs were treated with T-1840383 at the indicated concentration for 1 hour. Phosphorylation of VEGFR2 (Tyr1175), Akt (Ser473), and ERK1/2 (Thr202/Tyr204) following VEGF stimulation was analyzed by Western blotting. Western blotting was done in quadruplicate with very similar results. B, HUVECs were cocultured with fibroblasts. T-1840383 was added to the medium followed by VEGF stimulation. Shown are representative images after endothelial cell-specific staining (*n* = 4). C, total filament length and number of branch points of the stained tube-like structures were measured using image analysis software in 4 to 10 different fields for each condition. Columns, mean; bars, SD.
**T-1840383 inhibits VEGF receptor-2 phosphorylation and VEGF signaling in HUVECs**

Because T-1840383 also potently inhibits VEGFRTKs, the effects on VEGF signaling were tested in endothelial cells. As shown in Fig. 2A, T-1840383 inhibited VEGF-induced phosphorylation of VEGFR-2 (IC\textsubscript{50} = 0.86 nmol/L). It also blocked VEGF-induced activation of the downstream effectors ERK1/2 and AKT in a concentration-dependent manner. Consistent with this, T-1840383 inhibited VEGF-driven HUVEC proliferation with an IC\textsubscript{50} value of 1.8 nmol/L (Fig. 1E). When cocultured with fibroblasts, HUVECs formed capillary-like structures in response to VEGF, which was, however, abolished by the presence of T-1840383 (Fig. 2B). T-1840383 inhibited the VEGF-induced increase in total length of tubes and number of branch points with an IC\textsubscript{50} value of 12 and 8.3 nmol/L, respectively (Fig. 2C).

**T-1840383 inhibits in vivo c-Met signaling and tumor growth in mouse xenograft models**

To assess the in vivo potency of T-1840383 for inhibiting c-Met activation, the level of c-Met phosphorylation in MKN45 tumors was measured at several time points following a single oral administration of T-1840383 (Fig. 3A). T-1840383 administration at a dose of 2 mg/kg resulted in substantial inhibition of c-Met phosphorylation from 2 to 8 hours, with full recovery by 24 hours. In addition, dose-dependent inhibition of c-Met, AKT, and ERK1/2 phosphorylation was observed 4 hours after T-1840383 administration (Fig. 3B). Pharmacokinetic analysis showed that the plasma concentration of T-1840383 increased in a dose-dependent manner (over the dose range, 1–20 mg/kg; Supplementary Table S1). The tumor concentration of T-1840383 was lower than that in plasma, but relatively sustained (Supplementary Fig. S4). T\textsubscript{max} in tumors was achieved between 2 and 8 hours after dosing. T\textsubscript{max} and the inhibition of c-Met phosphorylation in tumors were well correlated (Fig. 3A). In the MKN45 tumor xenograft mouse model, once-daily 2-week administration resulted in a dose-dependent inhibition of tumor growth with the T/C values of 57%, 30%, and 4% at doses of 0.5, 1.5, and 5 mg/kg, respectively (Fig. 3C). T-1840383 caused tumor regression at 15 mg/kg. Additional tumor models were used to assess the potency of T-1840383 against c-Met–dependent or independent tumors: EBC-1 cells expressed elevated levels of constitutively active c-Met and were highly sensitive to T-1840383 in vitro (Fig. 1F and Supplementary Fig. S3A), whereas COLO 205 cells showed no sign of constitutively active c-Met and were
Insensitive to T-1840383 in vitro (Fig. 1F and Supplementary Fig. S3A). U-87 MG cells are reported to express both HGF and c-Met, and that their proliferation and/or tumor growth are at least partially driven by the HGF/c-Met autocrine loop (26); in the present study, T-1840383 showed marked antitumor activities in a dose-dependent manner in c-Met–dependent EBC-1 and U-87 MG tumor models (Fig. 3D and E). The T/C values for EBC-1 tumors were 85% and 26% at 0.5 and 1.5 mg/kg, respectively, and the tumors regressed by 22% and 52% at 5 mg/kg and 15 mg/kg, respectively. The T/C values for U-87 MG tumors were 72%, 32%, and 10% at 0.5, 1.5, and 5 mg/kg, respectively, and the tumors were almost static at 15 mg/kg. In the c-Met–independent COLO 205 tumor model, T-1840383 showed slightly less potent but still substantial antitumor activity compared with the c-Met–dependent tumor models, where the T/C values were 30% and 13% at 5 mg/kg and 15 mg/kg, respectively (Fig. 3F). A slight but statistically significant difference was observed in the body weight between vehicle- and T-1840383–treated EBC-1 tumor xenograft model mice at 15 mg/kg (2.3% loss vs. 7.9% gain compared with their initial body weight). However, T-1840383 was generally well tolerated at the efficacious doses without obvious signs of animal discomfort in all subcutaneous tumor xenograft models.

**T-1840383 inhibits tumor angiogenesis**

To further understand the mechanism of tumor growth inhibition by T-1840383, especially in c-Met independent tumor models, tumor angiogenesis was analyzed. A single oral administration of T-1840383 at 2 mg/kg resulted in a significant decrease in phosphorylated VEGFR-2 levels in the tumors of ectopically VEGFR-2-expressing 293 cells (Supplementary Fig. S5), suggesting that T-1840383 inhibits VEGFR activation at the efficacious doses. We next tested the antiangiogenic activities in tumor xenografts of the in vitro T-1840383 insensitive cell line A549. Following a 3-day treatment, T-1840383

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**Figure 4.** Effects of T-1840383 on tumor microvessel density, tumor cell proliferation, and tumor cell apoptosis in A549 tumors. A, mice bearing A549 tumors were treated with T-1840383 at 0.5, 1.5, and 5 mg/kg/day or vehicle for 3 days. Tumors were fixed, paraffin-embedded, sectioned, and immunostained for CD31. Scale bars, 400 μm. B, quantitative analysis of CD31-positive endothelial cells were conducted. C, tumor specimens were immunostained with anti-Ki67 antibody, and Ki67-positive, proliferating cells were quantified. D, apoptotic tumor cells were visualized and quantified by TUNEL assay. Column, mean (n = 4 or 5); bars, SEM. *P ≤ 0.025 versus the vehicle control group by the one-tailed Williams test.
exhibited significant antitumor activities with T/C values of 64%, 51%, and 35% at 0.5, 1.5, and 5 mg/kg, respectively (data not shown). T-1840383 treatment resulted in a dose-dependent reduction in the microvessel density in viable regions of the tumors by 29%, 50%, and 65% at 0.5, 1.5, and 5 mg/kg, respectively (Fig. 4A and B). In similar dose-dependent manners, T-1840383 treatment also led to a decreased tendency of cell mitosis (Ki67) and a significant increase in apoptosis (TUNEL) in the A549 tumor xenografts (Fig. 4C and D). Together, these results suggest that targeting tumor angiogenesis is one of the mechanisms by which T-1840383 inhibits growth of c-Met–independent tumors in vivo.

Antitumor activities of T-1840383 in a gastric tumor peritoneal dissemination model

To extend the finding that T-1840383 potently inhibits the growth c-Met–dependent cancer cells, T-1840383 was further evaluated in a mouse model of peritoneal dissemination using c-Met–dependent gastric cancer NUGC-4-luc cells. In this model, tumor growth was monitored noninvasively with bioluminescence emitted from NUGC-4-luc cells in mice. As shown in Fig. 5A and B, vehicle-treated mice showed a significant increase in tumor burden over the monitoring time from day 14 to day 41 (1.26 × 10^7/s vs. 3.63 × 10^7/s, P < 0.01). In contrast, the T-1840383–treated mice did not show a statistically significant increase in tumor burdens (1.30 × 10^7/s vs. 2.02 × 10^7/s). In addition, T-1840383 treatment resulted in a prolonged survival of mice compared with vehicle treatment (Fig. 5C, median survival: 46.5 days vs. 108.5 days, P < 0.01). In vehicle-treated mice, on the basis of in vivo imaging analysis, one mouse apparently became tumor-free and showed an exceptionally longer survival than the others, probably due to rejection of the tumor challenge.

c-Met expression and activation under hypoxia

Hypoxia, which is known to be a major stimulator of VEGF and consequent angiogenesis, has been shown to promote invasive tumor growth by transcriptional activation of c-met and by sensitizing cells to HGF stimulation (27). To explore this finding in our systems, we investigated c-Met expression and HGF-induced cell invasion in response to hypoxia. Hypoxia upregulated c-Met expression.
expression compared with normoxia (Fig. 6A). Moreover, we found that hypoxia induced c-Met phosphorylation in A549 cancer cells without HGF stimulation, whereas serum starvation had no such effects on c-Met expression and activation. Similar effects were seen in Caki-1 renal cell carcinoma, SUIT-2 pancreatic cancer, and U-87 MG glioma cells (Fig. 6B). In addition, hypoxia augmented SUIT-2 cell invasion induced by HGF compared with normoxia (Fig. 6C), suggesting functional relevance for the c-Met pathway in cancer cell invasive growth under hypoxia.

Discussion

Here, we have characterized T-1840383, a novel multi-targeted kinase inhibitor, in various functional assays. T-1840383 potently blocked c-Met activation and downstream signaling in cancer cells, whereas cell proliferation assay revealed that T-1840383 selectively inhibited cancer cells that harbored c-met gene amplification. Recent studies have shown that some gastric and lung cancer types harboring c-met amplification are "addicted" to the constitutively activated c-Met signals and that targeting such activated c-Met with small-molecule inhibitors results in marked inhibition of cell growth and survival (3, 28). Thus, the response profile of T-1840383 may well reflect the pharmacologic consequence of inhibition of c-Met rather than other targets. However, T-1840383 failed to display antiproliferative activities against some c-met–amplified cancer cells despite the fact that they expressed constitutively activated c-Met, suggesting that c-Met amplification/activation alone may not necessarily determine the response. Such resistance may be attributed to collateral activation of HER family members (29, 30), amplification of wild-type KRAS (31), or c-Met mutations that affect the inhibitor binding (32, 33), although these speculations need to be further investigated. The response profile we observed here may provide a tool to help identify reliable predictive markers, sensitive patient populations for T-1840383 or other c-Met inhibitors currently under clinical evaluation (reviewed in refs. 2, 7–9), and effective combination therapy for specific patients in the clinical setting.

We have also shown the in vivo antitumor efficacies of T-1840383. In the tumor xenograft models using in vitro T-1840383–sensitive cancer cells, T-1840383 treatment resulted in potent tumor growth inhibition, in agreement with attenuation of c-Met phosphorylation levels and...
downstream signaling in the tumors, suggesting that such potent efficacies primarily depend on direct inhibition of c-Met–driven tumor cell growth. In clinical settings, patients with gastric cancers having scirrhus-type stroma particularly showed poor prognosis even after curative resection; they also showed highly progressed peritoneal dissemination (34). This type of cancer has been observed to often possess c-met gene amplification (35). T-1840383 showed a potent antitumor efficacy in a peritoneal tumor dissemination model for a c-met–amplified gastric cancer, leading to a survival benefit in those mice. These results provide a rationale for further investigation of T-1840383 as an antitumor agent for patients with such types of gastric cancer.

In addition, our findings indicate that in vivo efficacy may also depend on the antiangiogenic properties. Indeed, T-1840383 potently inhibited endothelial cell responses to VEGF stimuli in vitro and decreased tumor microvessel density in vivo at the efficacious doses, even in the c-Met–independent tumor models. It is therefore clear that T-1840383 affects tumor angiogenesis as a VEGFR inhibitor. In addition, the c-Met inhibitory activity may contribute to angiogenesis inhibition because HGF/c-Met signaling induces tumor angiogenesis by directly inducing proliferation and migration of endothelial cells, by inducing expression of VEGF, as well as by downregulating thrombospondin 1, a negative regulator of angiogenesis (36). T-1840383 has a strong inhibitory effect on other angiokinases such as Tie-2 and PDGFR and thus such inhibitory activities may also be involved in the antiangiogenic property of T-1840383. We believe that inhibition of angiogenesis represents a mechanism by which T-1840383 manifests a wide spectrum of its antitumor activities. Indeed, compared with the selective inhibitors of VEGFR or c-Met, T-1840383 showed a broad in vivo antitumor spectrum (Supplementary Table S2).

Several small-molecule inhibitors, including cobanotinib/XL184 (37) and foretinib/XL880 (38), which simultaneously target c-Met and VEGFRs have been previously reported. Cobanotinib seems to have a stronger inhibitory activity against VEGFR2 than against c-Met (e.g., IC_{50} values: 0.035 vs. 1.3 nmol/L, respectively), whereas T-1840383 has very similar activities against them (2.2 vs. 1.9 nmol/L). Similar to T-1840383, foretinib has similar inhibitory activities against VEGFR2 and c-Met (0.4 vs. 0.86 nmol/L). While foretinib is required to be administered at a dose of approximately 100 mg/kg to effectively suppress c-Met and VEGFR activity in vivo, T-1840383 suppresses their activities in similar in vivo models at much lower doses (around 2 mg/kg). This finding may be attributed to more potent cellular activities and/or favorable pharmacokinetic profile of T-1840383.

Hepatocyte growth factor is a potent inducer of epithelial–mesenchymal transition (EMT; ref. 39) that facilitates most of the invasive growth functions of cancer, such as loss of adhesive junctions, motility, angiogenesis, and survival (reviewed in ref. 40). Thus, the ability of T-1840383 to block HGF-induced cell scattering and invasion may have important implications for treatment of the invasive growth and metastasis of cancer, although this remains to be confirmed experimentally. In the tumor xenograft models we used here, the HGF/c-Met paracrine loop was nearly absent due to the low affinity of mouse HGF derived from host stromal cells to the human c-Met on implanted cancer cells (41). It is therefore necessary to use relevant preclinical models, such as tumor allograft models or xenograft models in human HGF-transgenic or knock-in mice (42), where the HGF/c-Met paracrine loop is functional between stroma and cancer cells.

We further investigated c-Met signaling in response to hypoxia because hypoxia has been reported to promote invasive growth by transcriptional activation of c-Met (27). In agreement with previous studies on this subject, hypoxia indeed upregulated c-Met expression and promoted HGF-induced cell invasion. Moreover, we found that hypoxia elevated c-Met phosphorylation levels even in the absence of HGF stimuli, although this may be partly explained through the recent findings that hypoxia promotes interaction between c-Met and neuropilin-1, a coreceptor for VEGF-A, which in turn facilitates VEGF-induced c-Met activation (43). These results indicate a key role of c-Met activation in response to the hypoxic stress of cancer cells. Importantly, it has been suggested that hypoxia generated by inhibition of angiogenesis triggers pathways that make tumors more aggressive and metastatic (44, 45). Thus, it is possible that c-Met activation under hypoxia is a mechanism of refractoriness to antiangiogenic treatment by inducing a tumor-invasive switch (46). In addition, a recent study suggests that an alternative angiogenic pathway, via c-Met activation in endothelial cells, is involved in resistance/low-responsiveness to antiangiogenic treatment (47). Collectively, pharmacologic intervention of c-Met signaling is a potential strategy to prevent tumors from progressing under hypoxia, an adverse tumor microenvironment condition, and to circumvent resistance to angiogenesis inhibitors. This strategy clearly warrants further exploration.

In summary, T-1840383 is a novel, highly potent inhibitor of c-Met and VEGFR tyrosine kinases. Through this unique spectrum of dual kinase inhibition, T-1840383 showed in vivo broad and potent antitumor activities by targeting tumor epithelial cells and vasculature. Our findings indicate the therapeutic potential of targeting both c-Met and VEGFRs simultaneously with a single small-molecule inhibitor for treatment of various human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Writing, review, and/or revision of the manuscript: K. Nakamura, A. Hori
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Miyamoto, H. Miki, A. Hori
Other: Design and synthesis of T-1840383: S. Imamura
Study supervision: A. Hori

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References


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