Inhibition of c-Met and prevention of spontaneous metastatic spreading by the 2-indolinone RPI-1

Giuliana Cassinelli,[1] Cinzia Lanzi,[1] Giovanna Petrangolini,[1] Monica Tortoreto,[1] Graziella Pratesi,[1] Giuditta Cuccuru,[1] Diletta Laccabue,[1] Rosanna Supino,[1] Sara Belluco,[2] Enrica Favini,[1] Anna Poletti,[1] and Franco Zunino[1]

[1]Department of Experimental Oncology and Laboratories, Preclinical Chemotherapy and Pharmacology Unit, Istituto Nazionale Tumori; and [2]Department of Veterinary Pathology, University of Milan, Milan, Italy

Abstract

Hepatocyte growth factor (HGF) and its tyrosine kinase receptor Met play a pivotal role in the tumor metastatic phenotype and represent attractive therapeutic targets. We investigated the biochemical and biological effects of the tyrosine kinase inhibitor RPI-1 on the human lung cancer cell lines H460 and N592, which express constitutively active Met. RPI-1-treated cells showed down-regulation of Met activation and expression, inhibition of HGF/Met-dependent downstream signaling involving AKT, signal transducers and activators of transcription 3 and paxillin, as well as a reduced expression of the proangiogenic factors vascular endothelial growth factor and basic fibroblast growth factor. Cell growth in soft agar of H460 cells was strongly reduced in the presence of the drug. Furthermore, RPI-1 inhibited both spontaneous and HGF-induced motility/invasiveness of both H460 and human endothelial cells. Targeting of Met signaling by alternative methods (Met small interfering RNA and anti-phosphorylated Met antibody intracellular transfer) produced comparable biochemical and biological effects. Using the spontaneously metastasizing lung carcinoma xenograft H460, daily oral treatment with well-tolerated doses of RPI-1 produced a significant reduction of spontaneous lung metastases (−75%; $P < 0.001$, compared with control mice). In addition, a significant inhibition of angiogenesis in primary s.c. tumors of treated mice was observed, possibly contributing to limit the development of metastases. The results provide preclinical evidence in support of Met targeting pharmacologic approach as a new option for the control of tumor metastatic dissemination. [Mol Cancer Ther 2006;5(9):2388–97]

Received 5/3/06; revised 6/22/06; accepted 7/11/06.

Grant support: Ministero della Salute, Ministero Istruzione Università e Ricerca (Fondo per gli Investimenti della Ricerca di Base project), and Associazione Italiana per la Ricerca sul Cancro.

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.

Requests for reprints: Cinzia Lanzi, Istituto Nazionale Tumori, via Venezian 1, 20133 Milan, Italy. Phone: 39-02-23902627; Fax: 39-02-23902692. E-mail: cinzia.lanzi@istitutotumori.mi.it

doi:10.1158/1535-7163.MCT-06-0245

Introduction

Met tyrosine kinase represents the high-affinity receptor for hepatocyte growth factor (HGF), also known as scatter factor, expressed predominantly in epithelial and endothelial cells. The HGF/Met-mediated signaling is involved in physiologic processes, such as epithelial cell dissociation (scattering), invasion, branching morphogenesis, and angiogenesis (1, 2). Met receptor displays a heterodimeric structure consisting of an entirely extracellular 50-kDa α-subunit and a membrane-spanning 140-kDa β-subunit that are linked by disulfide bonds. The interaction of HGF with Met stimulates the tyrosine kinase activity of the receptor β-subunit, leading to autophosphorylation of specific tyrosine residues within the intracellular region. Such event further enhances the receptor enzymatic activity and allows the recruitment and phosphorylation of downstream signaling molecules and adaptor proteins. The phosphorylative events, in turn, lead to the activation of several signaling cascades, which regulate cell proliferation, survival, and cytoskeleton functions (3, 4).

Met activity can be deregulated through different mechanisms in human tumors, such as gene amplification, mutation, or involvement in HGF-dependent autocrine loops (5). The identification of germ-line missense mutations in the majority of hereditary papillary renal cell carcinomas provided an unequivocal evidence implying Met directly in tumorigenesis (6). Met is frequently overexpressed in human cancers (7). Moreover, recently, it has been shown that under hypoxic conditions, often present in solid tumors, Met is up-regulated and HGF/Met-dependent signaling is amplified (8). The overexpression or misexpression of HGF and/or Met has been correlated with poor prognosis in different tumor types, including lung cancer (5, 9, 10). Indeed, HGF and Met represent the main mediators of the ''invasive growth,'' an integrated set of cellular responses, including survival, proliferation, cell-cell dissociation, migration, and matrix degradation. Such a complex program, occurring physiologically during organ development and regeneration, becomes instrumental in tumor invasion and metastasis (11). In addition, Met activation has been implicated in the regulation of tumor neovascularization. This function, which is probably achieved through the coordination of different mechanisms promoting endothelial cell growth, motility, and invasion, involves both a direct HGF stimulation of Met expressed

on endothelial cells (12, 13) and an indirect regulation of paracrine angiogenic factors expressed by tumor cells (14, 15). Together, these observations suggest that inappropriate Met signaling may be crucial in cancer progression, thus representing an attractive therapeutic target.

We recently described the activity of the 2-indolinone RPI-1 as an inhibitor of Ret tyrosine kinase oncoproteins in human thyroid carcinoma cells and tumor xenografts expressing a RET oncogene mutant (16–18). The effects of RPI-1 on other tyrosine kinase targets and tumor models remain to be investigated in detail. In the present study, we have explored the biochemical and cellular effects of RPI-1 in two human lung carcinoma cell lines expressing a constitutively active Met receptor. In addition, taking advantage of the ability of the H460 non–small cell lung cancer (NSCLC) to develop spontaneous lung metastases in mice (19), the antimetastatic activity of RPI-1 was investigated also *in vivo*. The results indicate Met as an additional target of RPI-1 and support the interest for a pharmacologic approach aimed to target HGF/Met-dependent metastatic spreading.

Materials and Methods

Cell Culture and Drug Treatment Conditions. The human NSCLC cell line NCI-H460 and the SCLC cell line NCI-N592 (hereafter named H460 and N592, respectively) were kindly provided by Dr. A.F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). Cells were maintained routinely in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum. The human umbilical vascular endothelial cell (HUVEC) was routinely grown on BD BioCoat Matrigel Cellware (BD Biosciences, Bedford, MA) in Clonetics endothelial cell basal medium (Cambrex BioScience, Walkersville, MD) added with 10% fetal bovine serum and endothelial cell growth supplements provided by Cambrex BioScience. HUVECs from passages 6 to 9 were used for experiments.

The synthesis and the chemical structure of RPI-1 [1,3-dihydro-5,6-dimethoxy-3-[(4-hydroxyphenyl)methylene]-H-indol-2-one], formerly Cpd1, were reported previously (20). The drug was dissolved in DMSO and further diluted in cell culture medium (final solvent concentration 0.5% even in control samples).

The anchorage-independent growth assay in soft agar was done as described previously (20). The size and the number of colonies were determined by ImageMaster TotalLab version 1.10 (Amersham Biosciences, Little Chalfont, United Kingdom) analyzing digital images captured by ImageMaster VDS (Amersham Biosciences).

For biochemical analysis of HGF-induced effects, cells were serum starved for 24 hours, treated with RPI-1 for 18 hours, and then stimulated with HGF (20 ng/mL) for 10 minutes or 2 hours.

Antibody Intracellular Transfer. H460 cells were seeded at $4 \times 10^4/cm^2$ in six-well plates and, 24 hours later, subjected to protein delivery assay using PULSin (PolyPlus Transfection, Illkirch, France), a delivery reagent for the anti-phosphorylated Met (Tyr^{1234}/Tyr^{1235}) antibody (Upstate Biotechnology, Lake Placid, NY) or the rabbit IgG (Sigma, St. Louis, MO), according to the manufacturer's instructions. Cells were washed twice with saline solution before addition of the PULSin/antibody mixture. After 4 hours of incubation at 37°C, the antibody solution was removed and replaced with fresh complete medium. Cells were used for invasion assay 24 hours later.

Cell Migration and Invasion Assays. H460 cells or HUVECs were seeded in complete medium and treated with different concentrations of RPI-1 for 24 hours. Then, cells were harvested and transferred to 24-well Transwell chambers (Costar, Corning, Inc., Corning, NY) in serum-free medium.

For the migration assay, 1.2×10^5 cells per well were seeded in the upper Transwell chamber. The drug was added, at the same concentration used for the treatment, in both the upper and the lower chambers. HGF (20 and 30 ng/mL for H460 cells and HUVECs, respectively) was added in the lower chamber to assess HGF-induced migration. After 4 hours of incubation at 37°C, migrated cells were fixed in 95% ethanol, stained with a solution of 2% crystal violet in 70% ethanol, and counted under an inverted microscope.

For the invasion assay, the Transwell membranes were coated with 12.5 μg Matrigel per well (BD Biosciences, San Jose, CA) and dried for 1 hour. Cells, treated as described above, were transferred (2.4×10^5 per well) onto the artificial basement membrane. RPI-1 and HGF were added as indicated above. After 24 hours of incubation at 37°C, cells that invaded the Matrigel and migrated to the lower chamber were stained and counted as above.

ELISA. H460 cells were seeded at $2 \times 10^4/cm^2$ in complete medium and cultured for 2 days before serum starvation and treatment with solvent or RPI-1 for the indicated times. Then, conditioned media were harvested and clarified by centrifugation at 13,000 rpm for 15 minutes. Cells were trypsinized and counted. Supernatant aliquots were used for vascular endothelial growth factor (VEGF) protein determination with the human VEGF immunoassay kit from Biosource International (Camarillo, CA) according to the manufacturer's instructions. VEGF values were reported as $pg/mL/10^6$ cells.

RNA Interference. A pool of four c-Met-specific 21-nucleotides forming a 19-bp duplex core with 2-nucleotide 3′ overhangs was used to induce c-Met silencing, and a nonspecific control pool of small interfering RNA duplexes was used as negative control (Upstate Biotechnology). The oligonucleotides (100 nmol/L final concentration) were transfected into N592 cells using the siIMPORTER reagent (Upstate Biotechnology) according to the manufacturer's instructions. Cells were incubated for 120 hours before the preparation of cell lysates.

Immunoprecipitation and Immunoblot Analysis. Cells or tumor samples were processed for immunoprecipitation or total protein extraction and analyzed by immunoblotting as described previously (17). Tumor protein lysates were prepared from frozen tumor samples pulverized by the

Mikro-Dismembrator II (B. Brown Biotech International, Melsungen, Germany). Mouse monoclonal antibodies were as follows: anti-phosphotyrosine clone 4G10, anti-paxillin, and anti–basic fibroblast growth factor (bFGF) from Upstate Biotechnology; anti–protein kinase Bα/AKT from Transduction Laboratories (Lexington, KY); and anti-β-tubulin from Sigma. Rabbit polyclonal antibodies were as follows: anti-Met (C-12), anti–signal transducers and activators of transcription 3 (STAT3; K-15), and anti-VEGF (147) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphorylated Met (Tyr^{1234}/Tyr^{1235}); anti-phosphorylated AKT (Ser^{473}) and anti-phosphorylated STAT3 (Tyr^{705}) from Cell Signaling (Beverly, MA); anti-actin from Sigma; and anti-phosphorylated paxillin (Tyr^{31}) from Abcam Ltd. (Cambridge, United Kingdom).

The densitometric analysis of blots was done using ImageMaster TotalLab version 1.10.

In vivo Studies

Animals. All experiments were carried out using 8- to 11-week-old female athymic nude CD-1 mice (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy) according to the United Kingdom Coordinating Committee on Cancer Research Guidelines (21).

RPI-1 Formulation and Delivery. We reported previously the procedure to formulate RPI-1 at a maximum concentration of 5 mg/mL polysorbate 80 (20% of final volume) and a cold solution of 10% ethanol in distilled water under stirring on ice (18). The solution was administered by oral gavage at two dose levels (in a volume of 20–30 mL/kg of mouse body weight), b.i.d., by a prolonged daily schedule. Control mice were treated in parallel with the vehicle (polysorbate 80/ethanol/distilled water, 20:8:72).

H460 Tumor Model System. H460 cells were injected i.p. into nude mice, adapted to grow as ascites, and maintained *in vivo* by i.p. passages (5×10^6 per mouse in 0.5 mL PBS) as described previously (22). The effects of RPI-1 on the growth of primary tumors and spontaneous lung metastases were tested in mice inoculated s.c. in the right flank with H460 ascitic tumor cells (2×10^6 per mouse). Each control or drug-treated group included 9 to 11 mice. The growth of the s.c. tumor was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor weight and tumor weight inhibition percentage in treated over control mice were calculated as reported previously (17). Drug treatment was delivered by oral gavage for 45 days b.i.d. from day 0 at 100 and 150 mg/kg. At day 46, tumor-bearing mice were sacrificed by cervical dislocation and their lungs were removed. Lung lobes were spliced between two glass slides and the metastatic nodules were macroscopically counted against a bright light (19). Spontaneous lung metastases were present in 100% of control mice. Reading of metastases was done by two independent observers, with an interobserver reproducibility >95%. The metastatic nature of these areas was confirmed by histologic analysis of digital images obtained by Image Analysis System software (Delta System, Rome, Italy).

To induce experimental lung metastases, mice (12 per group) were injected i.v. with ascitic H460 cells (2×10^6 per mouse; ref. 23) and then treated with RPI-1 (100 mg/kg) b.i.d. for 42 days starting 1 hour after cell injection (day 0). At day 43, all mice were sacrificed for lung observation as described above. In the presence of confluent metastatic nodules, lungs were defined as tumor invaded and nodules were not counted. For ethical reasons, mice presenting signs of suffering were sacrificed before day 43.

For tumor angiogenesis determination, mice s.c. implanted with ascitic H460 tumor cells (four to five mice per group) were treated with RPI-1 at the dose of 150 mg/kg b.i.d. for 11 days starting the same day of cell inoculum. Six hours after the last treatment, mice were sacrificed and their tumors were excised. Half of each tumor was fixed in formalin for standard H&E staining, and the other half was fixed in zinc fixative for immunohistochemical analysis of microvessel density (MVD) by detection of the endothelium-specific adhesion molecule CD31 as described previously (24). Microvessels were quantified within six random fields at ×200 magnification (0.159 mm^2/field) using the Image Analysis System software. Scoring of tumor sections was done by two independent observers, with an interobserver reproducibility >95%.

For *in vivo* pharmacodynamic studies, mice implanted s.c. with H460 tumors were treated with oral vehicle or RPI-1 at 150 mg/kg b.i.d. 5 days weekly for 2 weeks. Four hours after the last administration, two mice per group were sacrificed and the resected tumors were snap frozen in liquid nitrogen before immunoblot analysis of tumor protein lysates (see above).

Statistical Analysis. The Student's *t* test was used to determine the significance of *in vitro* data or to compare tumor weight or MVD in control and treated mice. The number of metastases in control and treated mice was compared by the Mann-Whitney test, whereas the frequency of tumor-invaded lungs was compared using the χ^2 test.

Results

Inhibition of Met Tyrosine Phosphorylation and Expression in Human Lung Cancer Cell Lines. We first examined Met expression and activation in the N592 SCLC and H460 NSCLC cell lines. The two cell lines showed a different expression pattern of Met-related proteins, with the mature form of the protein ($p140^{Met}$) highly overexpressed only in H460 cells (Fig. 1A). Analysis of Met activation status in serum-starved cells showed that $p140^{Met}$ was constitutively phosphorylated in both cell lines, whereas the high molecular weight form, described as an uncleaved Met precursor (25), was phosphorylated only in N592 cells (Fig. 1B). Then, we investigated the ability of RPI-1 to inhibit the Met kinase activity in the two

cell lines following 24 hours of exposure (Fig. 1C and D). In N592 cells, RPI-1 induced a dose-dependent inhibition of tyrosine phosphorylation of both the $p140^{Met}$ and the uncleaved protein evidenced by immunoprecipitation. In addition, the reduced phosphorylation was associated with a down-regulation of the Met proteins. In H460 cells, RPI-1 strongly inhibited tyrosine phosphorylation of the Met mature form at all concentrations tested (15–60 μmol/L). The specific Met phosphorylation on Tyr^{1234}/Tyr^{1235}, known to activate the intrinsic kinase activity (3), was also examined in whole-cell lysates. A dose-dependent inhibition of Met phosphorylation was confirmed in both cell lines being evident around a drug concentration of 7.5 μmol/L. Nevertheless, differently from N592 cells, in H460 cells the overall Met expression was not affected by 24 hours of treatment with RPI-1.

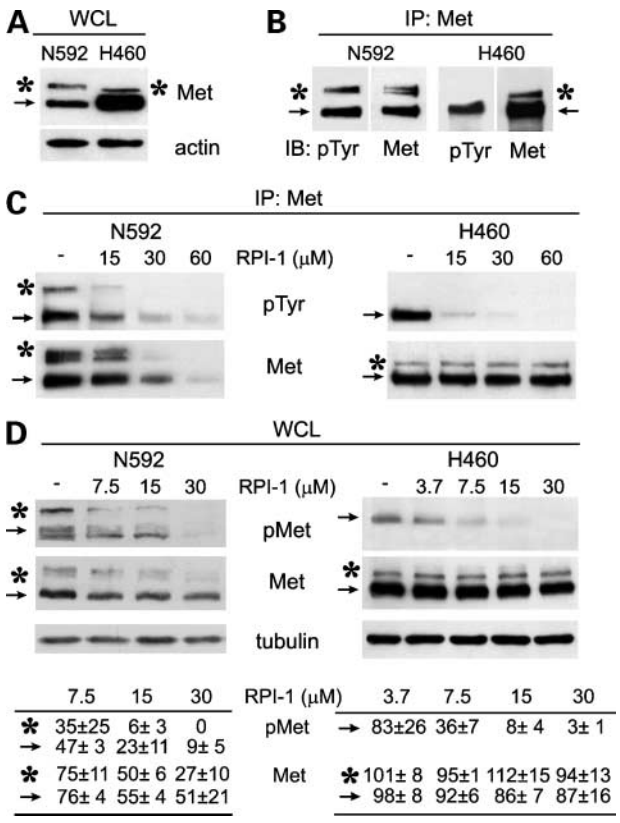

Figure 1. Effects of RPI-1 (24 h of exposure) on Met tyrosine phosphorylation and expression in the N592 SCLC and the H460 NSCLC cell lines analyzed by immunoblotting. **A,** Met and actin expression analyzed on whole-cell lysates (*WCL*). **B,** constitutive tyrosine phosphorylation of Met immunoprecipitated (*IP*) from serum-starved cells. **C,** effects of RPI-1 on Met tyrosine phosphorylation and expression. Met was immunoprecipitated from cell extracts and subjected to anti-phosphotyrosine (*pTyr*) immunoblotting. **D,** effects of RPI-1 on Met kinase-activating phosphorylation in N592 and H460 cells. Whole-cell lysates were analyzed using an anti-phosphorylated Met (Tyr^{1234}/Tyr^{1235}) antibody (*pMet*). The filters were then reblotted with anti-Met antibody after stripping. *Arrow,* mature form of Met. *Asterisk,* Met precursor that could be evidenced in N592 cells as a doublet depending on the length of the electrophoretic run. The phosphorylated form of the precursor corresponds to the upper band of the doublet. Anti-tubulin blots as controls for protein loading. The densitometric analysis was done on blots from two independent experiments. The intensity of the bands was normalized with respect to tubulin and expressed as percentage (mean ± SD) of the respective control. The blots are representative of at least two independent experiments.

Inhibition of HGF-Induced Met Activation and Downstream Events in H460 Cells. To investigate RPI-1 effects on HGF-dependent cellular events, the growth factor was added for 10 minutes or 2 hours to the medium of serum-deprived H460 cells exposed to different concentrations of RPI-1 for 18 hours (Fig. 2A). HGF-induced Met tyrosine phosphorylation was inhibited by RPI-1 in a dose-dependent way, and the inhibition was still evident 2 hours after the ligand addition. Similarly, HGF-induced activation of both AKT and STAT3 was inhibited even under conditions of marked activation of these Met signaling transducers (3). In such conditions, a weak drug-induced down-regulation of Met expression was also appreciable.

The biochemical effects of a prolonged exposure (72 hours) to RPI-1 were investigated in cells grown in complete medium. Figure 2B showed evidence of a marked drug-induced inhibition of Met tyrosine phosphorylation. Such inhibitory effect was associated with receptor down-regulation (not evidenced after 24 hours of treatment), indicating that, in the Met-overexpressing H460 cells, this event was delayed and required a prolonged drug exposure. A dose-dependent inhibition of AKT and STAT3 activation by RPI-1 was also observed. In addition, in the same experimental conditions, we observed inhibition of tyrosine phosphorylation of paxillin, a focal adhesion protein described previously as a target of Met signaling in lung cancer cells (26).

Inhibition of Anchorage-Independent Cell Growth and Cell Motility/Invasion. Increased tumorigenicity, motility, and invasiveness have been described as biological consequences of HGF/Met deregulation in tumor cells (5). In *in vitro* cell cultures, HGF/Met signaling has been reported to promote anchorage-independent growth, a feature associated with the malignant phenotype (27). We thus examined the ability of H460 cells to form colonies in soft agar in the presence of RPI-1. Treatment with 20 μmol/L RPI-1, a concentration inhibiting Met activation (Fig. 1C and D) and H460 cell proliferation by ~50% (IC_{50} = 24.5 ± 0.5 μmol/L, after 72 hours of treatment in a cell counting assay), resulted in a strong reduction of both colony number and size (Fig. 3). Indeed, in RPI-1-treated samples, colony number was reduced by 80%, the largest colonies (>1,000 pixels) disappeared, the medium colonies (300–1,000 pixels) were markedly reduced, and the small colonies (<300 pixels) represented 95% of the entire population. Similar results were obtained with N592 cells (data not shown).

Because Met activation plays a pivotal role in regulating cell motility and invasiveness of both tumor and endothelial cells (11, 12), we investigated the effects of RPI-1 on these processes in either H460 cells or HUVECs. In migration and invasion assays, pretreatment of carcinoma cells (15 and 30 μmol/L) prevented HGF-induced migration and inhibited, in a dose-dependent way, both spontaneous and HGF-induced invasion (Fig. 4A). The

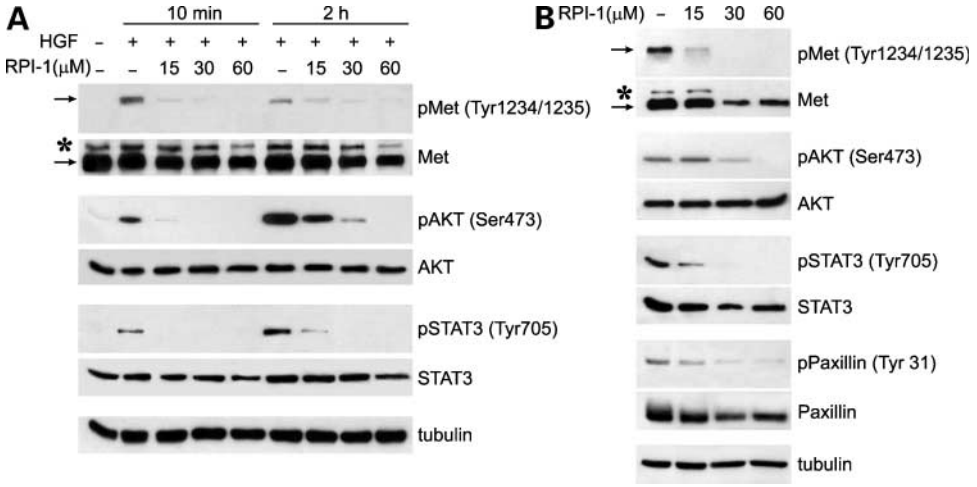

Figure 2. Effects of RPI-1 on Met-regulated signaling in H460 cells. **A,** inhibition of HGF-dependent signaling. Serum-starved cells were preincubated in the presence of solvent (–) or increasing concentrations of RPI-1 for 18 h and then stimulated with HGF for 10 min or 2 h. Whole-cell lysates were subjected to immunoblotting using antibodies recognizing the activated forms of the proteins Met, AKT, and STAT3. Stripped membranes were then reprobed with antibodies directed against the respective proteins. **B,** effect of prolonged RPI-1 treatment. Cells maintained in complete medium were incubated in the presence of solvent (–) or increasing concentrations of RPI-1 for 72 h. Cell extracts were analyzed by immunoblotting with the indicated antibodies. *Arrow,* mature form of Met. *Asterisk,* Met precursor. Anti-tubulin blots as controls for protein loading.

ability of the drug to interfere with these events *in vitro* was also confirmed in HUVEC, in which HGF-mediated migratory and invasive stimuli were prevented even by lower RPI-1 concentrations (3.75 and 7.5 μmol/L; Fig. 4B). These results thus supported that targeting Met by RPI-1 resulted in inhibition of HGF/Met-regulated biological functions related to the cell invasive phenotype.

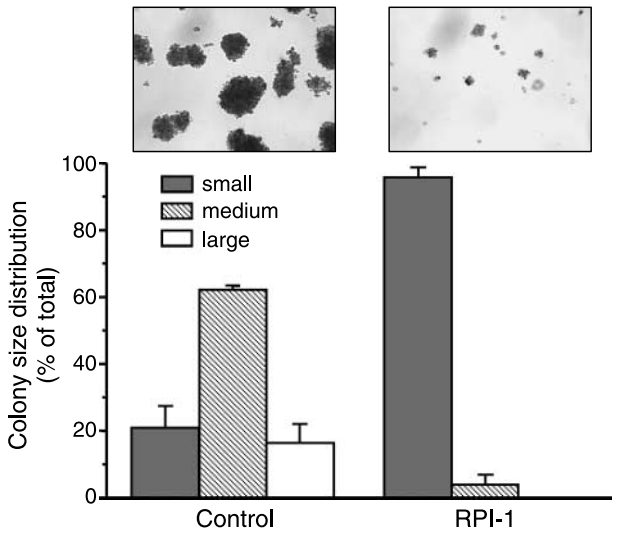

Figure 3. Inhibition of anchorage-independent cell growth. H460 cells were seeded in soft agar in the presence or absence of 20 μmol/L RPI-1. After 7 d, colony number and size were determined by computer image analysis. Representative images of control and treated colonies. Original magnification, ×100. Colony size distribution scored as percentage of small (<300 pixels), medium (300–1,000 pixels), or large (>1,000 pixels) colonies. *Columns,* mean of two independent experiments; *bars,* SE.

Because RPI-1 showed an inhibitory effect even on the spontaneous cell invasiveness, we investigated the role of Met activation in this process using an alternative approach for targeting Met signaling. The anti-phosphorylated Met (Tyr^{1234}/Tyr^{1235}) antibody recognizing activated Met was introduced in H460 cells by the protein delivery technique. As shown in Fig. 4C, the antibody intracellular transfer induced a significant inhibition of the spontaneous cell invasion in Matrigel (53.5% versus control cells; $P < 0.0001$). On the contrary, delivery of an aspecific antibody (IgG) into the cells did not interfere with the process. Consistent with the inhibition of Met signaling, cells loaded with the anti-phosphorylated Met antibody showed reduced AKT activation (data not shown). These findings confirmed that targeting Met signaling resulted in inhibition of the *in vitro* invasive potential of H460 cells.

***In vivo* Antimetastatic Activity of RPI-1.** To determine whether the inhibitory activity of RPI-1 on *in vitro* HGF/Met-dependent processes was reflected in *in vivo* reduction of tumor cell invasiveness, we examined the drug effect on the occurrence of spontaneous lung metastases in mice implanted s.c. with H460 cells, which are endowed with metastatic ability in nude mice (19). Two dose levels of RPI-1 were investigated (100 and 150 mg/kg) and delivered b.i.d. by oral gavage from days 0 to 45. The available model did not allow noninvasive assessment of therapeutic efficacy, and the use of bioluminescent imaging would be desirable in the future (28). When mice were sacrificed for lung observation (day 46), in RPI-1-treated mice, the mean number of macroscopic metastases was significantly reduced versus controls at both tested doses (Table 1). A clear dose-dependent effect was observed, with a reduction in the metastases number of 57% ($P < 0.05$) and 75% ($P < 0.001$) for the doses of 100 and 150 mg/kg, respectively. In

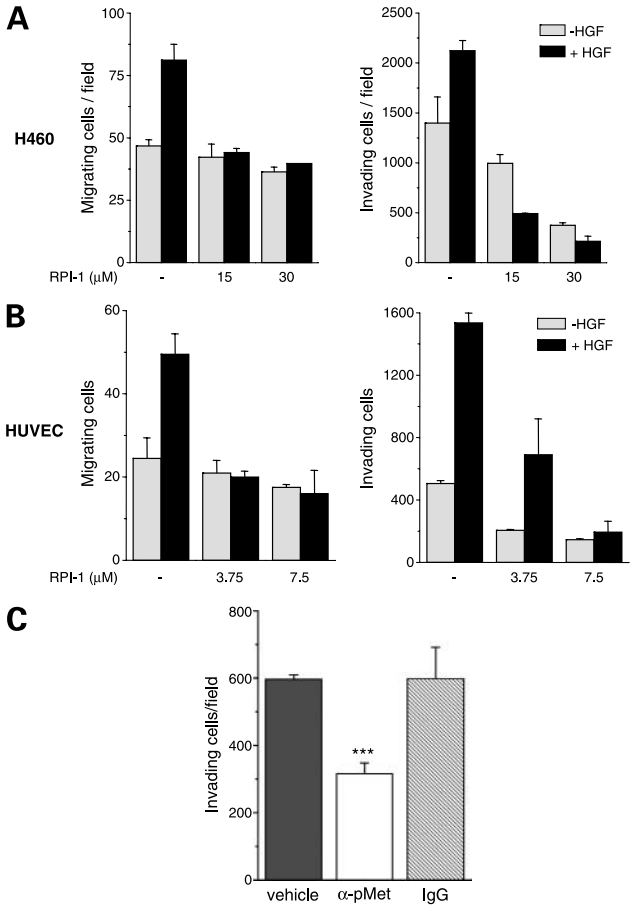

Figure 4. Cell migration and Matrigel invasion assays. **A** and **B,** effects of RPI-1 (24 h of exposure) on spontaneous and HGF-induced migration and Matrigel invasion of H460 cells (**A**) and HUVECs (**B**). Cells were subjected to the migration assay or the invasion assay in serum-free medium with or without exogenous HGF. Migrating or invading cells are reported as number of H460 cells per field or number of total HUVECs counted under light microscope. *Columns,* mean of three replicates; *bars,* SD. One experiment, representative of three. **C,** inhibition of spontaneous Matrigel invasion of H460 cells by anti-phosphorylated (Tyr^{1234}/Tyr^{1235}) Met antibody (*α-pMet*). Serum-starved cells exposed to the delivery reagent (*vehicle*), anti-phosphorylated (Tyr^{1234}/Tyr^{1235}) Met antibody, or aspecific immunoglobulins (*IgG*) were subjected to the invasion assay. *Columns,* mean of three replicates expressed as invading cells per field; *bars,* SD. One experiment, representative of three. ***, $P < 0.0001$ versus vehicle-treated cells by Student's *t* test.

addition, one treated mouse (100 mg/kg) was metastasis-free when macroscopically inspected and showed evidence of small tumor nodules only at the histologic analysis. At the microscopic observation, H&E staining indicated that lungs of control mice were characterized by a high amount of neoplastic nodes (>100 neoplastic cells each node), whereas in lungs of RPI-1-treated mice only a moderate invasion, arranged in small nodules of neoplastic cells (<50 cells), was evident (Fig. 5A). In contrast to the remarkable antimetastatic effect, the inhibitory activity of RPI-1 on the primary s.c. growing tumor was marginal (Table 1) and only a 31% of tumor weight inhibition ($P > 0.05$ versus control tumors) was achieved by the higher dose after 45 days of treatment, indicating that the strong inhibition of spontaneous lung metastases in treated mice could not be ascribed to a reduced primary tumor burden. Consistently, no linear relation was found ($r = 0.5$, by the Spearman rank correlation test) between s.c. tumor weight and number of spontaneous lung metastases (data not shown). The body weight loss values never exceeded 15% during the entire experimental frame, and lethal toxicity due to drug treatments was never observed.

To assess Met targeting *in vivo*, the receptor activation status was examined in tumor lysates. Immunoblot analysis showed a reduced Met autophosphorylation and, accordingly, a reduced activation of AKT in tumors derived from RPI-1-treated mice (150 mg/kg b.i.d. 5 days weekly for 2 weeks), thus confirming the drug pharmacodynamic activity (Fig. 5B).

To get insight into the steps of the metastatic process affected by RPI-1, we examined the effects of the drug on artificial metastases (i.e., the tumor lung colonization induced by i.v. injection of H460 ascitic cells), thereby bypassing the first steps of the metastatic cascade. Mice were treated with oral RPI-1 (100 mg/kg b.i.d. for 42 days) and, the day after the last dose, sacrificed for lung observation. At this time, in the control group, 2 of 12 mice had already been euthanized and presented tumor-invaded lungs, 9 mice showed one or more lobes totally invaded by metastases, and only 1 mouse presented countable metastatic foci in all lung lobes. In the RPI-1-treated group, all mice were alive at the end of the experiment, 8 of 12 mice presented tumor-invaded lungs, and 4 mice (33% versus 8% in controls) presented countable metastatic areas. However, no statistical significance versus control mice was achieved ($P > 0.2$, by χ^2 test), indicating a moderate effect of RPI-1 in this assay compared with the effect obtained on the spontaneous metastatic process.

Table 1. Effects of RPI-1 on the growth of primary s.c. tumor and spontaneous lung metastases of the NSCLC H460 xenograft

Dose (mg/kg)*	TWI (%)†	Metastases‡		
		Incidence (%)§	Mean ± SE‖	Inhibition (%)¶
Solvent	—	100	32 ± 8	—
100	0	90	14 ± 3.6	57**
150	31	100	8 ± 1.6	75††

Abbreviation: TWI, tumor weight inhibition.
*Treatments were delivered orally b.i.d. for 45 days. Solvent: polisorbate 80/ethanol/distilled water (20:8:72).
†Tumor weight inhibition percentage in treated over control mice at day 45.
‡Observation time: day 45.
§Percentage of mice with metastases on total number of mice (9 mice in the solvent-treated group; 11 and 9 mice in the groups receiving 100 and 150 mg/kg RPI-1, respectively).
‖Mean number of metastatic nodules macroscopically counted in each lung.
¶Percentage of inhibition in treated over control mice.
**$P < 0.05$ versus controls, by Mann-Whitney test.
††$P < 0.001$ versus controls, by Mann-Whitney test.

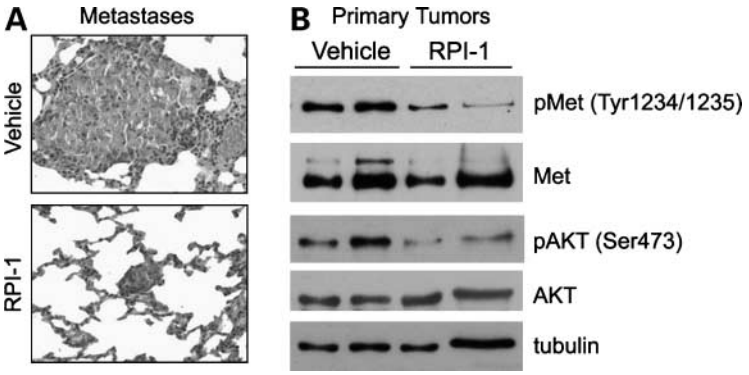

Figure 5. Effects of RPI-1 (orally, 150 mg/kg, b.i.d.) on H460 s.c. growing primary tumors and lung metastases in nude mice. **A,** representative lung sections showing a tumor node (⩾100 cells) and a small nodule (<50 cells) in the parenchyma of vehicle- or RPI-1-treated mice (for 45 d). H&E staining. Original magnification, ×200. **B,** inhibition of Met and AKT activation in H460 s.c. growing tumors. Tumor-bearing mice were treated with vehicle or RPI-1 (5 d weekly for 2 wks). Tumors were then removed and processed for immunoblot analysis. The specific antibodies were used to detect the activating phosphorylation of Met and AKT. After stripping, filters were reprobed with antibodies recognizing Met or AKT protein. Anti-tubulin blot as loading control. Representative blots of three separate experiments.

Antiangiogenic Effects of RPI-1. Because angiogenesis is regulated by HGF/Met through different mechanisms (12, 13, 29), the effect of oral RPI-1 treatment (150 mg/kg b.i.d. for 11 days) on tumor neovascularization was investigated in primary s.c. growing H460 tumors. Tumor angiogenesis, analyzed by immunohistochemical detection of MVD, appeared effectively reduced, being the MVD of treated tumors significantly lower compared with that of control tumors (50% inhibition; $P < 0.0001$; Fig. 6A).

To elucidate the cellular bases of such an effect, we examined *in vitro* the production of proangiogenic growth factors by tumor cells exposed to RPI-1. A reduced expression of two major endothelial growth factors, VEGF and bFGF, was observed in whole-cell extracts from RPI-1-treated H460 and N592 cells (Fig. 6B). VEGF levels were then detected in the conditioned medium of H460 cells exposed to solvent or RPI-1 by an ELISA test. An increase in VEGF concentration in the medium from 24 to 48 hours of control cultures indicated the release of the angiogenic factor (Fig. 6C). In the medium from treated cells, a significant dose-dependent reduction of VEGF was evidenced compared with the controls (33.5% and 50.4% of inhibition after exposure to 30 and 40 μmol/L RPI-1, respectively). In accordance to a previous report (30), bFGF was barely detectable in the conditioned medium of H460 cells (data not shown).

Next, the RNA interference strategy (31) was used as an alternative method for disrupting Met signaling. Because this approach did not produce significant effects on Met expression in H460 cells (data not shown), the small interfering RNA methodology was applied to silence the receptor, expressed at a lower level, in the N592 cells (Fig. 6D). A pool of four RNA duplexes specifically targeting Met mRNA caused the disappearance of the receptor precursor and an appreciable reduction of the mature form in N592 cells. Such selective Met silencing resulted in the abrogation of AKT activation and in an almost complete down-regulation of VEGF and bFGF expressions. These findings confirmed the regulatory role of Met on the two proangiogenic factors in this cell system and supported the specificity of RPI-1 effects as a consequence of Met signaling inhibition.

Discussion

In the present study, we showed that the small-molecule tyrosine kinase inhibitor RPI-1 inhibited Met activation and signaling in lung carcinoma cell lines both *in vitro* and *in vivo*. Indeed, oral treatment with RPI-1 resulted in a significant antimetastatic effect and inhibition of angiogenesis in the human H460 NSCLC xenograft. We showed that treatment with RPI-1 produced a marked and dose-dependent inhibition of spontaneous lung metastases in mice bearing s.c. growing H460 tumors. The reduction of tumor spreading could not be ascribed to the treatment effect on the primary tumor growth because only a marginal inhibition of the s.c. implanted tumor was achieved. Thus, the effect of RPI-1 on H460 tumor metastatic dissemination might reflect the inhibition of a predominant motogenic rather than proliferative/survival effect of HGF on tumor cells (32). Accordingly, no evidence of apoptosis induction could be detected in *in vitro*–treated cells (data not shown). Furthermore, in our tumor model, RPI-1 was more effective against the formation of lung metastases spontaneously spreading from the s.c. tumor xenograft than against artificial metastases produced by lung colonization of cancer cells directly injected into the vascular flow. Metastases from solid tumors represent the final outcome of a multistep process that involves cellular and molecular mechanisms existing in tumor cells and in cells or components of tumor microenvironment (33). Whereas spontaneous metastases occur only on completion of all steps of the process, artificial metastases do not need to overcome the barrier of the primary site microenvironment. Thus, it is likely that the remarkable inhibition of spontaneous lung metastasis formation might reflect a preferential drug effect on early steps of the metastatic cascade. Indeed, HGF and Met, as main mediators of *in vivo* ''invasive growth,'' actively participate to early steps by promoting critical events, such as dissociation of tumor cells at the primary site, invasion through the basement membrane and stroma, motility, angiogenesis, and interaction between tumor and endothelial cells (34). Because microenvironment could affect the first steps of the process, the study in tumor models orthotopically growing might provide additional information on the therapeutic potential of the inhibitor.

The results of our *in vitro* experiments indicated that biological functions relevant to the invasive/metastatic phenotype of lung cancer cells were impaired by RPI-1 treatment. In fact, in H460 cells, drug concentrations able to inhibit Met activation prevented HGF-induced cell migration and inhibited both spontaneous and HGF-dependent invasion of Matrigel. Because migration through the artificial extracellular matrix depends on the cell ability to degrade the matrix and undergo chemotaxis, the inhibitory effect of RPI-1 in the cell invasion assay is likely relevant to its antimetastatic activity. The role of Met inhibition in the observed RPI-1 effects was supported by the similar effects obtained by targeting Met signaling *in vitro* with an antibody recognizing the activated Met. Furthermore, the inhibition of Met-mediated biological effects was consistent with the down-modulation of signaling pathways involving AKT, STAT3, and paxillin observed in RPI-1-treated cells. Indeed, although it cannot be ruled out the possibility that inhibition of additional tyrosine kinases might contribute to these effects, it is worth noting that paxillin and phosphatidylinositol 3-kinase, which lies upstream to AKT, have been implicated in cell migration and cytoskeleton functions regulated by Met (4, 35, 36). In addition, our data show a marked inhibition of H460 cell anchorage-independent growth in soft agar, an *in vitro* feature of cancer cells that has been related to STAT3 activation through Met signaling (27). Constitutive activation of Met in other NSCLC cells has been found to correlate with their ability of growing in the absence of anchorage, thus suggesting a role of the receptor in the promotion of cell survival in conditions where adhesion is lost, such as during dissemination from the primary tumor site (37).

A further contribution to the reduced formation of spontaneous metastases in treated mice was possibly due to the drug inhibitory effect on the Met regulatory function

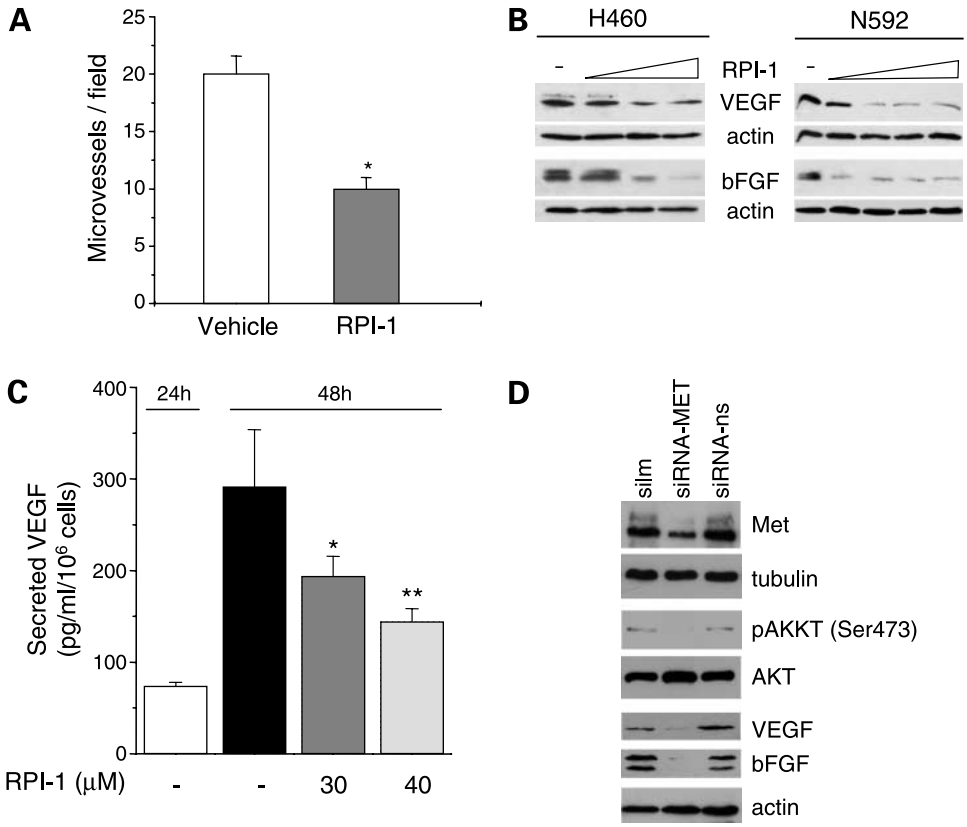

Figure 6. Antiangiogenic effects of RPI-1. **A,** reduction of MVD in H460 s.c. tumor xenografts in nude mice. Tumor-bearing mice were treated with vehicle or RPI-1 (150 mg/kg b.i.d. for 11 d). Tumors were fixed in zinc and processed for immunohistochemical staining of $CD31^+$ endothelial cells. *Columns,* mean (four to five tumors per group); *bars,* SE. *, $P < 0.0001$ versus control tumors by Student's *t* test. **B,** *in vitro* down-regulation of proangiogenic factors in lung cancer cells. H460 cells were exposed to RPI-1 (15, 30, and 60 μmol/L) for 72 or 96 h for the expression analysis of VEGF or bFGF proteins, respectively. N592 cells were exposed to RPI-1 (7.5, 15, 30, and 60 μmol/L) for 72 h. Immunoblotting was done on total cell extracts. Anti-actin blot as control for protein loading. **C,** reduction of secreted VEGF in H460 cell culture. The amounts of the growth factor were determined by ELISA test in the conditioned medium of cells exposed to solvent for 24 or 48 h or to RPI-1 for 48 h. *Columns,* mean VEGF concentration done in triplicates; *bars,* SE. *, $P < 0.05$ versus 48 h of control; **, $P < 0.005$ versus 48 h of control. **D,** inhibition of Met signaling and proangiogenic factor expression in N592 cells by Met RNA interference. N592 cells were treated with the transfection reagent (*siIm*) or transfected with Met-specific RNA duplexes (*siRNA-MET*) or the nonspecific RNA pool (*siRNA-ns*). Immunoblot analyses were done on whole-cell lysates to detect Met expression, activated AKT, and AKT, VEGF, and bFGF proteins. Anti-tubulin and anti-actin blots as controls for protein loading. Pictures are representative of two (**A** and **B**) or three (**C** and **D**) independent experiments.

of angiogenesis processes and, hence, on the chance for tumor cells to reach the vascular flow (33). In fact, MVD was significantly decreased in s.c. growing H460 primary tumors of RPI-1-treated mice compared with control tumors. Such finding was not reflected in a substantial reduction of primary tumor growth, indicating that, in this model, the two events are not associated (38). Similar effects were obtained on N592 xenograft growth and angiogenesis (data not shown). The inhibition of Met in endothelial cells could contribute to the reduction of MVD because HGF is a potent angiogenic factor acting directly on these cells (12, 13). Accordingly, HGF-induced migration and invasion of HUVEC in *in vitro* assays was inhibited by RPI-1. In addition, our data showed a down-regulation of VEGF and bFGF in H460 and N592 cells treated with the drug. These results suggest that RPI-1 might also affect the paracrine amplification of angiogenesis promoted by angiogenic factors produced by the tumor cells. Because VEGF expression is positively regulated by HGF/Met in different cell systems (14, 15), the drug effect on VEGF was in keeping with the inhibition of Met signaling. Moreover, down-regulation of bFGF has been observed even in N592 cells where Met was silenced by RNA interference. These findings supported the interpretation that RPI-1-induced down-modulation of proangiogenic factors is a consequence of Met targeting.

In addition, to prevent Met tyrosine phosphorylation, RPI-1 induced a down-regulation of Met protein expression. Such an effect was also observed on Ret proteins (17, 18). Further studies are needed to elucidate such RPI-1 property associated with protein tyrosine kinase inhibition, which is shared with other ATP-competitive inhibitors (39, 40). The results of the present study suggest that Met down-regulation is dependent on the basal protein expression level because a prolonged exposure to the drug (72 versus 24 hours) was needed to observe a reduction in the highly overexpressing H460 cells compared with N592 cells. Nonetheless, on stimulation by exogenous HGF, the reduction of Met expression detectable in H460 cells even after 18 hours of RPI-1 treatment suggests that the drug could promote a mechanism involved in the physiologic ligand-induced receptor down-regulation (41).

Different strategies have been developed to explore the therapeutic efficacy of targeting the HGF/Met regulatory system (2, 7, 42, 43), although a clinically useful therapeutic option has yet to be found. *In vitro* anti-invasive activity and/or *in vivo* cytoreductive antitumor activity have been described by chemical Met inhibitors, including indolinone compounds (44–49). An *in vivo* inhibitory effect against tumor dissemination and angiogenesis has been shown previously by peptide antagonists of HGF/Met delivered or not by a gene therapy approach (34, 50, 51). The present study, by the use of a spontaneous lung-metastasizing human tumor model, provides evidence that a marked *in vivo* antimetastatic and antiangiogenic activity can be achieved even by a pharmacologic approach with a small-molecule Met inhibitor. Thus, RPI-1, known as an inhibitor of Ret tyrosine kinases (16–18, 20), likely functions as a multitarget agent similarly to other *in vivo* effective tyrosine kinase inhibitors (52, 53).

Collectively, the data presented in the study provide preclinical support to the view that Met targeting by a pharmacologic approach holds promise as an exploitable option in antimetastatic therapy of cancer.

Acknowledgments

We thank the Medicinal Chemistry Department of Cell Therapeutics, Inc., Europe (Bresso, Italy) for the synthesis of RPI-1, Laura Zanesi for help in editing the article, and Tommaso Dragani for helpful discussion.

References

1. Birchmeier C, Gherardi E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol 1998;8:404–10.

2. Jiang WG, Martin TA, Parr C, Davies G, Matsumoto K, Nakamura T. Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. Crit Rev Oncol Hematol 2005;53:35–69.

3. Furge KA, Zhang YW, Vande Woude GF. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene 2000; 19:5582–9.

4. Comoglio PM. Pathway specificity for Met signaling. Nat Cell Biol 2001; 3:E161–2.

5. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility, and more. Nat Rev Mol Cell Biol 2003;4:915–25.

6. Schmidt L, Duh FM, Chen F, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet 1997;16:68–73.

7. Maulik G, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. Cytokine Growth Factor Rev 2002;13: 41–59.

8. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the *met* protooncogene. Cancer Cell 2003;3:347–61.

9. Takanami I, Tanana F, Hashizume T, et al. Hepatocyte growth factor and c-Met/hepatocyte growth factor receptor in pulmonary adenocarcinomas: an evaluation of their expression as prognostic markers. Oncology 1996;53:392–7.

10. Masuya D, Huang C, Liu D, et al. The tumor-stromal interaction between intratumoral c-Met and stromal hepatocyte growth factor associated with tumour growth and prognosis in non-small-cell lung cancer patients. Br J Cancer 2004;90:1555–62.

11. Comoglio PM, Trusolino L. Invasive growth: from development to metastasis. J Clin Invest 2002;109:857–62.

12. Bussolino F, Di Renzo MF, Ziche M, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 1992;119:629–41.

13. Grant DS, Kleinman HK, Goldberg ID, et al. Scatter factor induces blood vessel formation *in vivo*. Proc Natl Acad Sci U S A 1993; 90:1937–41.

14. Dong G, Chen Z, Li Z-Y, Bancroft CC, Van Waes C. Hepatocyte growth factor/scatter factor-induced activation of MEK and P13K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. Cancer Res 2001;61:5911–8.

15. Zhang Y-W, Su Y, Volpert OV, Vande Woude GF. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. Proc Natl Acad Sci U S A 2003; 100:12718–23.

16. Lanzi C, Cassinelli G, Cuccuru G, et al. Inactivation of Ret/Ptc1 oncoprotein and inhibition of papillary thyroid carcinoma cell proliferation by indolinone RPI-1. Cell Mol Life Sci 2003;60:1449–59.

17. Cuccuru G, Lanzi C, Cassinelli G, et al. Cellular effects and antitumor activity of RET inhibitor RPI-1 on MEN2A-associated medullary thyroid carcinoma. J Natl Cancer Inst 2004;96:1006–14.

18. Petrangolini G, Cuccuru G, Lanzi C, et al. Apoptotic cell death induction and angiogenesis inhibition in large established medullary thyroid

carcinoma xenografts by Ret inhibitor RPI-1. Biochem Pharmacol 2006; 72:405 – 14.

19. Corti C, Pratesi G, De Cesare M, et al. Spontaneous lung metastases in a human lung tumor xenograft: a new experimental model. J Cancer Res Clin Oncol 1996;122:154 – 60.

20. Lanzi C, Cassinelli G, Pensa T, et al. Inhibition of transforming activity of the ret/ptc1 oncoprotein by a 2-indolinone derivative. Int J Cancer 2000;85:384 – 90.

21. Workman P, Twentyman P, Balkwill F, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCR) guidelines for the welfare of animals in experimental neoplasia. 2nd ed. Br J Cancer 1998;77:1 – 10.

22. Pratesi G, Manzotti C, Tortoreto M, Audisio RA, Zunino F. Differential efficacy of flavone acetic acid against liver versus lung metastases in a human tumour xenograft. Br J Cancer 1991;63:71 – 4.

23. De Cesare M, Pratesi G, Veneroni S, Bergottini R, Zunino F. Efficacy of the novel camptothecin gimatecan against orthotopic and metastatic human tumor xenograft models. Clin Cancer Res 2004;10:7357 – 64.

24. Petrangolini G, Pratesi G, De Cesare M, et al. Antiangiogenic effects of the novel camptothecin ST1481 (gimatecan) in human tumor xenografts. Mol Cancer Res 2003;1:863 – 70.

25. Kermorgant S, Zicha D, Parker PJ. Protein kinase C controls microtubule-based traffic but not proteasomal degradation of c-Met. J Cell Biol Chem 2003;278:28921 – 9.

26. Maulik G, Kijima T, Ma PC, et al. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. Clin Cancer Res 2002;8: 620 – 7.

27. Zhang Y-W, Wang L-M, Jove R, Vande Woude GF. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene 2002; 21:217 – 26.

28. Dikmen ZG, Gellert GC, Jackson S, et al. *In vivo* inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. Cancer Res 2005;65:7866 – 73.

29. Martin TA, Parr C, Davies G, et al. Growth and angiogenesis of human breast cancer in a nude mouse tumour model is reduced by NK4, a HGF/SF antagonist. Carcinogenesis 2003;8:1317 – 23.

30. Ogawa T, Takayama K, Takakura N, Kitano S, Ueno H. Anti-tumor angiogenesis therapy using soluble receptors: enhanced inhibition of tumor growth when soluble fibroblast growth factor receptor-1 is used with soluble vascular endothelial growth factors receptor. Cancer Gene Ther 2002;9:633 – 40.

31. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol 2003;4: 457 – 67.

32. Yi S, Chen J-R, Viallet J, Schwall RH, Nakamura T, Tsao M-S. Paracrine effects of hepatocyte growth factor/scatter factor on non-small-cell lung carcinoma cell lines. Br J Cancer 1998;77:2162 – 70.

33. Liotta LA, Stetler-Stevenson WG. Principles of molecular cell biology of cancer: cancer metastasis. In: DeVita VT, Hellman S, Rosenberg SA, editors. Cancer principles & practice of oncology. Philadelphia: JB Lippincott Co.; 1993. p. 134 – 49.

34. Matsumoto K, Nakamura T. NK4 (HGF-antagonist/angiogenesis inhibitor) in cancer biology and therapeutics. Cancer Sci 2003;4:321 – 7.

35. Bardelli A, Basile ML, Audero E, et al. Concomitant activation of pathways downstream of Grb2 and PI 3-kinase is required for MET-mediated metastasis. Oncogene 1999;18:1139 – 46.

36. Liu Z-X, Yu CF, Nickel C, Thomas S, Cantley LG. Hepatocyte growth factor induces ERK-dependent paxillin phosphorylation and regulates paxillin-focal adhesion kinase association. J Biol Chem 2002;277: 10452 – 8.

37. Qiao H, Hung W, Tremblay E, et al. Constitutive activation of Met kinase in non-small-cell lung carcinomas correlates with anchorage-independent cell survival. J Cell Biochem 2002;86:665 – 77.

38. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 2002;94:883 – 93.

39. Carniti C, Perego C, Mondellini P, Pierotti MA, Bongarzone I. PP1 inhibitor induces degradation of RETMEN2A and RETMEN2B oncoproteins through proteasomal targeting. Cancer Res 2003;63:2234 – 43.

40. Citri A, Alroy I, Lavi S, et al. Drug-induced ubiquitylation and degradation of ErbB receptor tyrosine kinases: implications for cancer therapy. EMBO J 2002;21:2407 – 17.

41. Jeffers M, Taylor GA, Weidner KM, Omura S, Vande Woude GF. Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol Cell Biol 1997;17:799 – 808.

42. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions, and potential for therapeutic inhibition. Cancer Metastasis Rev 2003;22: 309 – 25.

43. Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett 2005;225:1 – 26.

44. Morotti A, Mila S, Accornero P, Tagliabue E, Ponzetto C. K252a inhibits the oncogenic properties of Met, the HGF receptor. Oncogene 2002;21:4885 – 93.

45. Christensen JG, Schreck R, Burrows J, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. Cancer Res 2003;63:7345 – 55.

46. Sattler M, Pride YB, Ma P, et al. A novel small molecule Met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase. Cancer Res 2003;63:5462 – 9.

47. Wang X, Le P, Liang C, et al. Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. Mol Cancer Ther 2003;2:1085 – 92.

48. Jagadeeswaran R, Ma PC, Seiwert TY, et al. Functional analysis of c-Met/hepatocyte growth factor pathway in malignant pleural mesothelioma. Cancer Res 2006;66:352 – 61.

49. Ma PC, Schaefer E, Christensen JG, Salgia R. A selective small molecule c-MET inhibitor, PHA665752, cooperates with rapamycin. Clin Cancer Res 2005;11:2312 – 9.

50. Michieli P, Mazzone M, Basilico C, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. Cancer Cell 2004;6:61 – 73.

51. Mazzone M, Basilico C, Cavassa S, et al. An uncleavable form of pro-scatter factor suppresses tumor growth and dissemination in mice. J Clin Invest 2004;114:1418 – 32.

52. Shawver LK, Slamon D, Ullrich A. Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell 2002;1:117 – 23.

53. Broxterman HJ, Georgopapadakou NH. New cancer therapeutics: target-specific in, cytotoxics out? Drug Resist Update 2004;7: 79 – 87.

Molecular Cancer Therapeutics

Inhibition of c-Met and prevention of spontaneous metastatic spreading by the 2-indolinone RPI-1

Giuliana Cassinelli, Cinzia Lanzi, Giovanna Petrangolini, et al.

Mol Cancer Ther 2006;5:2388-2397.