

Inhibition of the Met Receptor in Mesothelioma

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Abstract **Background:** Expression of the Met receptor and its ligand, hepatocyte growth factor (HGF), has been observed in 74% to 100% and 40% to 85% of malignant pleural mesothelioma (MPM) specimens, respectively. HGF stimulation has been shown to enhance MPM cell proliferation, migration, cell scattering, and invasiveness.

Experimental Design: To investigate a potential therapeutic role for the Met receptor in MPM, we examined the effects of PHA-665752, a specific small-molecule inhibitor of the Met receptor tyrosine kinase, in a panel of 10 MPM cell lines.

Results: Two of the cell lines, H2461 and JMN-1B, exhibited autocrine HGF production as measured by ELISA (3.9 and 10.5 ng/mL, respectively, versus <0.05 ng/mL in other cell lines). Evaluation of PHA-665752 across the 10 MPM cell lines indicated that despite Met expression in all cell lines, only in cell lines that exhibited a Met/HGF autocrine loop, H2461 and JMN-1B, did PHA-665752 inhibit growth with an IC₅₀ of 1 and 2 μmol/L, respectively. No activating mutations in Met were detected in any of the cell lines. Consistent with observed growth inhibition, PHA-665752 caused cell cycle arrest at G₁-S boundary accompanied by a dose-dependent decrease in phosphorylation of Met, p70S6K, Akt, and extracellular signal-regulated kinase 1/2. Growth of H2461 cells was also inhibited by neutralizing antibodies to HGF and by RNA interference knockdown of the Met receptor, confirming that growth inhibition observed was through a Met-dependent mechanism. PHA-665752 also reduced MPM *in vitro* cell migration and invasion.

Conclusions: Taken together, these findings suggest that inhibition of the Met receptor may be an effective therapeutic strategy for patients with MPM and provides a mechanism, the presence of a HGF/Met autocrine loop, by which to select patients for PHA-665752 treatment.

Malignant pleural mesothelioma (MPM) is a rare thoracic malignancy with ~3,000 new cases diagnosed annually in the United States. MPM consists of three major histologic subtypes: epithelial, sarcomatoid, and biphasic types (1). Despite the diverse therapeutic approaches, such as surgery, radiotherapy, chemotherapy, and/or their combinations, there is no proven curative modality and the median survival ranges from 6 to 18 months (2, 3). MPM is often considered a chemotherapy resistant disease and the response rates of commonly used single chemotherapeutic agents are all <25% (1, 4). Combination

chemotherapy with cisplatin/pemetrexed has recently emerged as a new standard systemic therapy based on a phase III trial (5). Despite this advance, most patients experience tumor progression or relapses within a year. Therefore, additional therapeutic approaches are still needed for patients with MPM.

Receptor tyrosine kinases are potential therapeutic targets for a variety of malignancies. MPM is known to aberrantly express a variety of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), Met, platelet-derived growth factor receptor, and vascular endothelial growth factor receptor (6–9). We have shown previously that the growth of only a minority of MPM cell lines *in vitro* is altered by an EGFR-selective tyrosine kinase inhibitor, gefitinib, or a dual EGFR/Erbb2 inhibitor, lapatinib (10, 11). Clinical studies of EGFR tyrosine kinase inhibitors in patients with MPM have also been disappointing. Both gefitinib and erlotinib have been studied in clinical trials of previously untreated patients with MPM and were associated with minimal antitumor activity (12, 13). These findings suggest that there may be other receptor tyrosine kinases that regulate MPM growth and be better candidates for useful therapeutic targets.

The Met receptor tyrosine kinase is a growth factor receptor with roles in both normal development and tumorigenesis. The ligand for the receptor, hepatocyte growth factor (HGF)/scatter factor (SF), was originally identified as both a potent mitogen for hepatocytes (HGF) and a fibroblast-derived cell motility factor (SF; refs. 14, 15). The biological consequences of HGF/SF-Met signal transduction include increased tumorigenicity,

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induction of cell motility, increased *in vitro* invasiveness, augmented *in vivo* metastases, increased production of proteases (urokinase plasminogen activator) capable of degrading the basal membrane/extracellular matrix, and increased angiogenesis (16–18). In primary tumors, Met expression has also been associated with a poor prognosis in patients with breast cancers and nasopharyngeal cancers (19, 20). Expression of both HGF and Met in tumor cells leads to constitutive activation through an autocrine loop, a mechanism that has been described in gliomas, osteosarcomas, and breast and prostate cancers (21, 22). Activating point mutations in the Met receptor have been described in a variety of human malignancies, including a minority of non-small cell and small cell lung carcinomas (23–28).

Met is virtually uniformly expressed in MPM. The expression of Met protein has been detected by immunohistochemistry in 74% to 100% of paraffin-embedded mesothelioma tumor specimens but not in normal mesothelial cells (6, 8). In addition, HGF/SF expression has been detected by immunohistochemistry in 40% to 85% of mesothelioma specimens (6, 8). Both Met and HGF/SF seem to be expressed in the tumor cells themselves, thus also suggesting an autocrine role in mesotheliomas. HGF/SF can also be detected by ELISA from the majority of pleural effusions obtained from patients with mesothelioma (29, 30). HGF stimulation of mesothelioma cell lines has been shown to increase their migration, invasiveness, proliferation, and adhesion and the synthesis of matrix metalloproteinases (29, 31–33). HGF/Met signaling may also have a role in the transformation of mesothelial cells to mesotheliomas. Cacciotti et al. (34) showed that mesothelial cells transfected with SV40 DNA, an oncogene with a putative role for MPM development, activate the HGF/Met autocrine loop and this is accompanied by a change to a fibroblast-like morphology and G₁-S cell cycle progression. Furthermore, exposure of crocidolite asbestos, a causative agent in mesothelioma, to rat pleural mesothelial cells increases Met expression, which is regulated by the early-response proto-oncogene *fra-1* (35). Taken together, these findings suggest that Met signaling, especially through autocrine activation, has important roles in various aspects of early development of mesothelioma biology and its inhibition may be therapeutically important.

In the present study, we examine the role of inhibition of HGF/Met signaling in MPM using PHA-665752, a small-molecule inhibitor for Met tyrosine kinase (36). We show that inhibition of HGF/Met signaling by PHA-665752 leads to cell cycle arrest at G₁-S boundary, decrease in cell growth, migration, and invasion, and increased cell-cell contact in two MPM cell lines that have a HGF/Met autocrine loop. We validate our findings with an independent method of inhibiting the Met receptor using RNA interference (RNAi) and also characterize the molecular effects of HGF/Met inhibition in MPM cells.

Materials and Methods

Cell culture. H2452 (sarcomatoid), H28 (sarcomatoid), H2052 (epithelial), and MSTO-211H (biphasic) MPM cells (37) were purchased from American Type Culture Collection, Manassas, VA. H2461 (epithelial), H2591 (epithelial) and H2373 (sarcomatoid) MPM cells were kindly provided by National Cancer Institute and have been characterized previously (38). JMN-1B (sarcomatoid) cells were kindly provided by Dr. James Rheinwald, Brigham and Women's Hospital, Boston, MA. MS428 (biphasic) and MS589 (biphasic) MPM cells were

kindly provided by Dr. Jonathan A. Fletcher, Brigham and Women's Hospital, Boston, MA (39, 40). All cells, except H2373, were maintained in RPMI 1640 (Cellgro; Mediatech, Inc., Herndon, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc., Woodland, CA), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L glutamine. H2373 cells were maintained in ACL-4 medium (Life Technologies, Inc., Rockville, MD) with 5% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L glutamine.

Drugs. PHA-665752 was a generous gift of Pfizer Pharmaceuticals, La Jolla, CA. Stock solutions were prepared in DMSO and stored at –20°C. The drugs were diluted in fresh medium before each experiment. In all experiments, the final concentration of DMSO was <0.1%.

Hepatocyte growth factor detection assay. HGF determination in the medium of MPM cells was done similarly to previously described methods (32). Briefly, confluent cells grown in serum-containing medium were washed with PBS and cultured in serum-free medium for 24 hours. The cells were then incubated in fresh serum-free medium for another 48 hours, the medium was collected and centrifuged, and the supernatant was stored at –70°C until analysis. For the determination of HGF, ELISA was done according to the manufacturer's recommended procedures (Human HGF Immunoassay, Quantikine, R&D Systems, Inc., Minneapolis, MN). All samples were run in triplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Antibodies and Western blotting. Cells were lysed in lysis buffer [20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10% glycerol, 1% NP40, and 0.42% NaF] containing proteinase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄ 2 µg/mL aprotinin, and 5 µg/mL leupetin). Proteins were separated by gel electrophoresis on 5% to 12% polyacrylamide gels selected depending on the molecular weight of the target, transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and detected by immunoblotting using an enhanced chemiluminescence system (New England Nuclear Life Science Products, Inc., Boston, MA). The Met (C-28), focal adhesion kinase (FAK; A-17), paxillin (H-114), and p27 (C-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phosphospecific Met (Tyr¹²³⁴/Tyr¹²³⁵), phosphorylated Akt (pS473), and phosphorylated p70S6K (Thr³⁸⁹) antibodies were purchased from Cell Signaling Technology, Beverly, MA. The phosphorylated extracellular signal-regulated kinase 1/2 (pT185/pY187), extracellular signal-regulated kinase 1/2, phosphorylated FAK (pY397), phosphorylated FAK (pY861), phosphorylated paxillin (pY31), and phosphorylated paxillin (pY118) antibodies were purchased from Biosource International, Inc. (Camarillo, CA). Anti-HGF-neutralizing antibody (MAB294) was purchased from R&D Systems.

Cell growth assay. MPM cells were plated in six-well tissue culture plates (50 × 10³–80 × 10³ per well) and cultured in ACL-4 or RPMI 1640 containing 1.5% fetal bovine serum overnight. The cell numbers for each line were determined empirically, so that the cells were not confluent by counting. Twenty-four hours following plating, the drugs were added (day 0). Cells from each well were collected using 1 mL trypsin and the cell number was counted with Coulter counter (Z1 Cell and Particle Counter, Beckman Coulter, Fullerton, CA) on day 5, except JMN-1B, which was assayed at day 3 due to its fast growth rate. Coulter counter was set to count cells that are >10 µm in size. The experiments were done in triplicate and on at least two separate occasions. Each data point represents the mean and SD (error bars). Trypan blue exclusion was used in preliminary studies to evaluate the effects on cell viability and we found no significant increase in cell death as a result of any of the treatments (data not shown).

The anti-HGF-neutralizing antibody was used at a final concentration of 1 µg/mL. This concentration is sufficient to neutralize the bioactivity of exogenous recombinant human HGF (100 ng/mL) by 70% on 4MBr-5 cells (R&D Systems). In a pilot growth study with H2461 examining the effects of the anti-HGF-neutralizing antibody (0.1 µg/mL–10 µg/mL),

no additional effects on growth were observed above 1 $\mu\text{g}/\text{mL}$; thus, this concentration was chosen for further analyses (data not shown).

Fluorescence-activated cell sorting analysis. The fluorescence-activated cell sorting analysis was done using methods similar to those described previously (11, 41). Briefly, 1×10^6 to 1.5×10^6 cells were seeded into 10-cm³ plates, and indicated drugs were added 24 hours later. The cells were then trypsinized and fixed overnight in ethanol at 4°C. Fixed cells were then resuspended in 500 $\mu\text{g}/\text{mL}$ RNase A (Sigma, St. Louis, MO), centrifuged, resuspended in 69 $\mu\text{mol}/\text{L}$ propidium iodine (Sigma) in 38 mmol/L sodium citrate, incubated at room temperature for 30 minutes, and analyzed by FACScan using CellQuest software (Becton Dickinson Labware, Franklin Lakes, NJ). All experiments were repeated thrice.

Cell morphology. Cells were plated on 60-mm plates (BD Falcon, Bedford, MA) and cultured in full serum medium. Twenty-four hours following plating, the drugs were added and cells were cultured for another 24 hours. The plates with subconfluent cells were photographed using an Olympus (Melville, NY) IX70 microscope with a DVC1310 digital video camera and a QED camera with Standalone 145 software (QED Imaging, Inc., Pittsburgh, PA).

Scratch wound migration assay. MPM cells were seeded in 60-mm tissue culture plates and cultured in full serum-containing medium until confluence. When the cells became confluent, a gap was created by scraping cells with P1000 pipette tips and cells were cultured in fresh full serum medium with or without 1 $\mu\text{mol}/\text{L}$ PHA-665752 or 1 $\mu\text{g}/\text{mL}$ anti-HGF-neutralizing antibody. The plates were photographed at 0, 4, 8, and 24 hours for JMN-1B cells and at 0, 24, 48, and 72 hours for the other cells using an Olympus IX70 microscope with a DVC1310 digital video camera and a QED camera with Standalone 145 software (QED Imaging). Cell migration distance was measured at three fixed positions (top, right middle, and bottom on the screen) using Adobe Photoshop version 5.5 measurement tool. Each data point represents the mean and SD (error bars).

Invasion assay. The BD BioCoat FluoroBlok Invasion System (Becton Dickinson, Bedford, MA) was used to assess cell invasion. The system consists of 24-multiwell plates with inserts containing Matrigel-coated membrane. The membrane has 8- μm pores and blocks the passage of light at wavelengths of 490 to 700 nm at 99% efficiency, so that one can differentiate between the light emitted by cells prelabeled with fluorescence on the upper surface from that on the lower surface. MPM cells (1.2×10^6 - 1.3×10^6) were seeded on a pair of 10-cm cell culture plates (Falcon) and allowed to grow in full serum medium. Twenty-four hours after plating, 1 $\mu\text{mol}/\text{L}$ PHA-665752 was added to one set of cells and were cultured for another 24 hours. Subsequently, the cells were labeled for 1 hour with 10 $\mu\text{g}/\text{mL}$ DiI (Molecular Probes, Eugene, OR) diluted in medium. The labeled cells were trypsinized and resuspended in full serum medium with or without 1 $\mu\text{mol}/\text{L}$ PHA-665752. Cells (1.5×10^5) were seeded on the surface of the inserts according to the manufacturer's instruction. The fluorescence was quantified from the bottom using CytoFluor 2300 plate reader (Millipore Corp., Bedford, MA) at 0, 4, 8, 24, 48, 72, and 96 hours at excitation and emission wavelengths of 530 and 590, respectively. The experiments were done in triplicate and on at least two separate occasions. Each data point represents the mean and SD (error bars).

Met RNA interference constructs. Two types of Met RNAi constructs were used in these studies. Three short hairpin RNA (shRNA) constructs targeting Met were obtained from the Harvard RNAi consortium. Each construct contained a 21-bp sequence targeting different regions of Met, a 6-nucleotide hairpin sequence (CTCGAG), and a 21-bp complementary strand sequence cloned into the pLKO.1puro vector. All clones were sequence verified. The regions of Met targeted (listed as the location of the first base pair of each oligonucleotide) and the forward primer sequences were as follows: Met 345 (5'-AGAATGTCATTCTA-CATGAGC-3'), Met 4293 (5'-ATGTGAACCGCTACTTATGTGC-3'), and Met 4571 (5'-ATCAGAACCAGGCTTGGTC-3'). The clones were transfected into MPM cell lines with LipofectAMINE (Invitrogen, Carlsbad, CA) using the manufacturer's recommended conditions and

selected in 1 $\mu\text{g}/\text{mL}$ puromycin-containing medium for 10 to 14 days. Individual clones were picked, expanded, and subjected to analyses.

An inducible Met RNAi system was also developed using the pSuperior vector system (Oligoengine, Seattle, WA). Oligomeric sequences with predicted RNAi activity against the c-Met mRNA were selected with the use of the Oligoengine software program. The oligomer 5'-GATCCCCGGACCGGTTTCATCAACTTCTCAAGAGA-GAAGTTGATGAACCGGTCTCTTTTGGAAA-3' and its complement 5'-AGCTTTTCCAAAAAGGACCGGTTTCATCAACTTCTCTCTTGAAGTTGATGAACCGGTCCGGG-3' were synthesized, phosphorylated, annealed, and cloned between the *Bgl*II and *Hind*III sites of pSuperior. The clones were verified by sequencing. To test the efficacy of the constructs, COS7 cells were transfected with 100 ng pcDNA encoding hemagglutinin epitope-tagged c-Met and 100 ng pcDNA- β -galactosidase with Fugene (Roche, Indianapolis, IN) at a 3:1 Fugene/DNA ratio. The cells were lysed 48 hours after transfection and the proteins were separated by electrophoresis and subjected to Western blotting. Expression of Met was detected with an anti-hemagglutinin antibody and complete Met knockdown was observed (data not shown). MPM cell lines were sequentially transfected with a Tet repressor construct (pcDNA6/TR; Invitrogen) and the pSuperior construct containing the Met RNAi. Cell lines were sequentially selected in blastacidin (5 $\mu\text{g}/\text{mL}$) or puromycin (1 $\mu\text{g}/\text{mL}$), and individual colonies were isolated, expanded, and used for subsequent experiments. Cell lines containing both constructs were grown with or without doxycycline (5 $\mu\text{g}/\text{mL}$) and the effects were assayed.

Met receptor sequencing. Total RNA was isolated from all MPM cell lines using Trizol (Invitrogen) and purified using RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA). cDNA was transcribed from ~2 μg total RNA with SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. PCR primers were designed with M13 tails to allow for direct sequencing. The details of the PCR conditions and the primers have been published previously (27).

Results

Expression of Met and hepatocyte growth factor in malignant pleural mesothelioma cell lines. Ten MPM cell lines were assayed for their expression of the Met receptor using Western blotting. As shown in Fig. 1, all MPM cells examined expressed the Met receptor. We next examined HGF production by MPM cells in cell culture medium using ELISA and found that two MPM cell lines, JMN-1B and H2461, secreted >70 times the amount of HGF than the other eight cells (Fig. 1). Given prior studies showing mutations in Met in other malignancies, we also sequenced the entire Met receptor from all 10 cell lines (23–27). No mutations were detected (data not shown).

Cell cycle distribution. To characterize the role of Met in regulating MPM cell cycle progression, we did cell cycle analysis

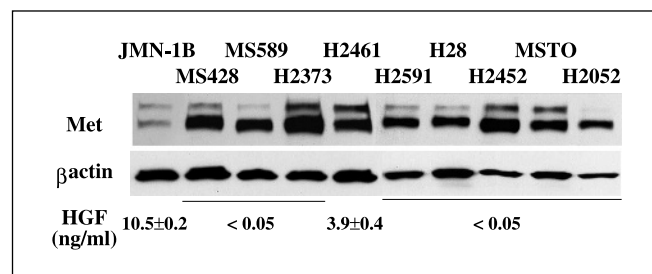


Fig. 1. Expression of Met and HGF in mesothelioma cell lines. Mesothelioma cell lines grown in full medium were lysed and immunoblotted for the Met receptor. The blot was stripped, with β -actin as loading control. HGF levels were measured in the supernatant and assayed by ELISA.

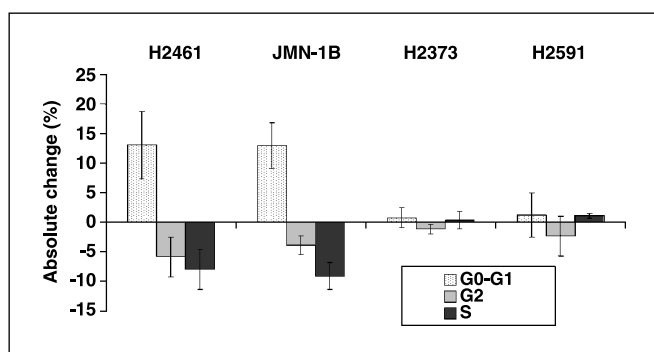


Fig. 2. Changes in cell cycle distribution following PHA-665752 treatment. Mesothelioma cell lines that do (H2461 and JMN-1B) or do not (H2373 and H2591) secrete HGF were treated with 1 $\mu\text{mol/L}$ PHA-665752 and cell cycle distribution was assayed as described in Materials and Methods. Columns, absolute changes compared with untreated control in each cell cycle phase; bars, SD. For G₀-G₁ phase, the absolute changes (mean \pm SD) are as follows: H2461, 13.1 \pm 5.7%; JMN-1B, 13.0 \pm 3.9%; H2373, 0.79 \pm 1.7%; and H2591, 1.2 \pm 3.7%.

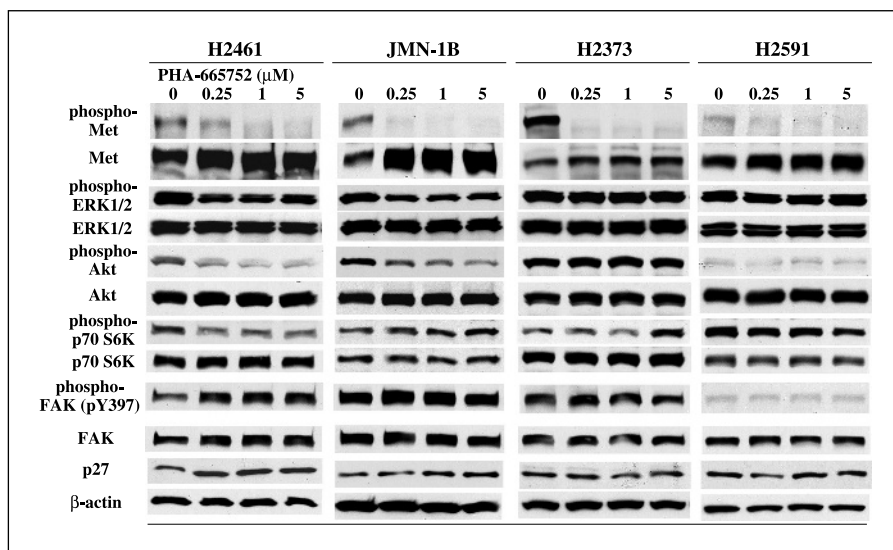
in 10 MPM cell lines with increasing concentrations of PHA-665752 (0-5 $\mu\text{mol/L}$). A marked effect of PHA-665752 on cell cycle distribution was only observed in H2461 and JMN-1B cells, the same lines that exhibited autocrine production of HGF (Fig. 2). In contrast, we observed no changes in cell cycle distribution on the other eight cell lines (Fig. 2; data not shown). In H2461 and JMN-1B cells, treatment with PHA-665752 led to increase in the G₁-G₀ fraction along with a decrease in the G₂-M and S fractions, indicating the cell cycle arrest at G₁-S boundary (Fig. 2). A plateau effect at which increased PHA-665752 concentrations had no additional effect on cell cycle distribution was reached at 1 $\mu\text{mol/L}$ (data not shown). No sub-G₁ fraction, consistent with apoptosis, was observed by treatment with PHA-665752 in either H2461 or JMN-1B (data not shown).

Based on these findings, we chose H2461 (epithelial) and JMN-1B (sarcomatoid) cell lines, which exhibited autocrine HGF production and G₁-S cell cycle arrest by PHA-665752, and H2373 (sarcomatoid) and H2591 (epithelial) cell lines, which did not produce HGF and were unaffected by PHA-665752 treatment (Fig. 2), for further experiments.

PHA-665752 inhibits Met signaling. The phosphorylation of the Met receptor and representative downstream signaling molecules was examined in MPM cells grown in full serum medium with increasing concentrations of PHA-665752 (0-5 $\mu\text{mol/L}$). Despite variations in the baseline levels of phosphorylated Met, PHA-665752 substantially inhibited the phosphorylation of Met receptor with relatively low concentrations (0.25 $\mu\text{mol/L}$) in all cell lines studied (Fig. 3). The total amount of Met was increased by PHA-665752 most notably in JMN-1B cells and but also in H2461, H2373, and H2591 cells. This is likely due to inhibition of Tyr¹⁰⁰³ (pY1003), the binding site for c-Cbl that mediates the ubiquitination of the Met receptor (36, 42, 43). PHA-665752 decreased phosphorylation of Akt, p70S6K, and extracellular signal-regulated kinase 1/2 in both H2461 and JMN-1B cells but not in H2373 or H2591 cell lines, with no change in total amount of these proteins. There was no decrease in phosphorylation of FAK (Tyr³⁹⁷) or paxillin (Tyr³¹; data not shown) in any mesothelioma cell lines tested. Interestingly, phosphorylated FAK and paxillin seemed to slightly increase with PHA-665752 treatment in H2461 and JMN-1B cells. To further investigate this phenomenon, we also examined the different phosphorylation sites of FAK and paxillin, pY861 and pY118, respectively, and the changes by the treatment with PHA-665752 were identical (data not shown). In addition, PHA-665752 also led to an increase in p27, a known mediator of G₁-S cell cycle arrest, in H2461 and, to a lesser degree, JMN-1B, both cell lines in which we observed G₁-S arrest (Fig. 2).

PHA-665752 inhibits cell growth of malignant pleural mesothelioma cells. To examine the effects of PHA-665752 on MPM cell growth, cultures were maintained in serum-containing medium with increasing concentrations of PHA-665752 (0-5 $\mu\text{mol/L}$) for 3 to 5 days and the cell number was evaluated using a Coulter counter. As seen in Fig. 4, PHA-665752 significantly inhibited cell growth in H2461 with IC₅₀ of 1 $\mu\text{mol/L}$ (Fig. 4A). PHA-665752 also caused growth inhibition of JMN-1B cells with a slightly higher IC₅₀ of 2 $\mu\text{mol/L}$ (Fig. 4A). In contrast, no significant effects on growth were observed in H2591 and H2373 until reaching the highest concentrations tested (5 $\mu\text{mol/L}$). Taken together, the growth inhibition of PHA-665752 is likely due to induction of G₁-S cell

Fig. 3. Effects of PHA-665752 on phosphorylation of Met and downstream signaling molecules. Mesothelioma cell lines were treated with increasing concentrations of PHA-665752 for 24 hours and the effects on phosphorylation of Met and downstream signaling molecules were examined using immunoblotting. The phosphorylation status of Met, extracellular signal-regulated kinase 1/2 (ERK1/2), Akt, p70S6K, and FAK was detected using phosphospecific antibodies for each protein. The blot was stripped and rehybridized with an antibody detecting the total form of the protein. The blot was stripped, with β -actin as loading control.



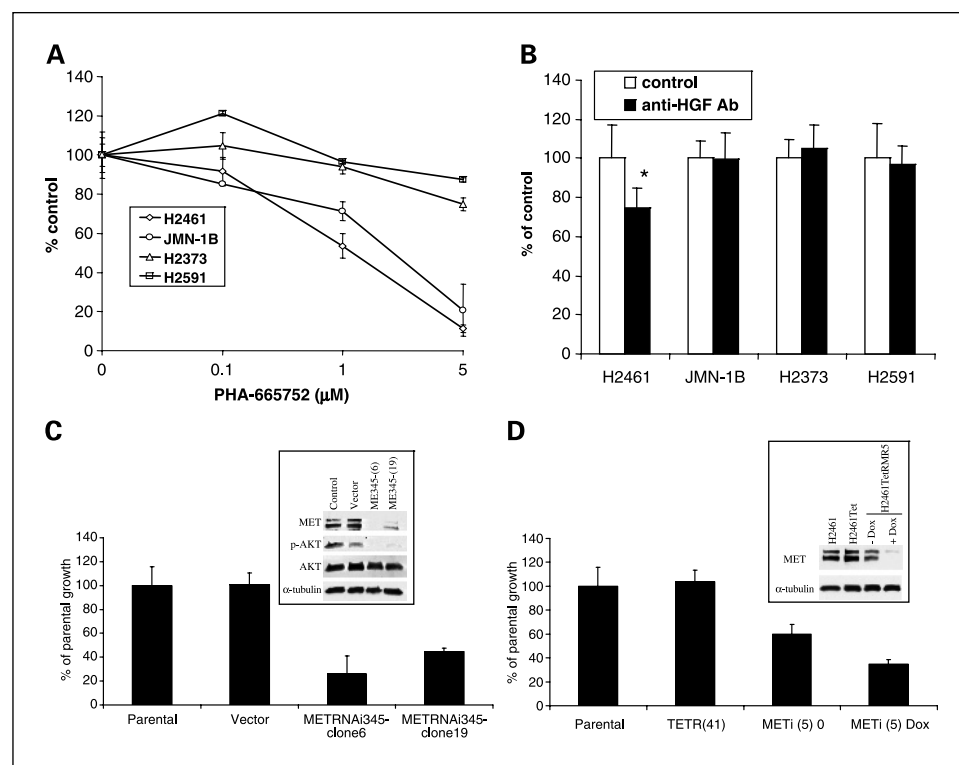


Fig. 4. Inhibition of Met causes growth inhibition in mesothelioma cell lines. **A**, mesothelioma cell lines were grown in the presence of increasing concentrations of PHA-665752 and the effect on growth was assayed. The IC_{50} s for H2461 and JMN-1B were 1 and 2 μ mol/L, respectively, but were not reached for H2373 and H2591. The differences in growth at 1 μ mol/L PHA-665752 compared with control are significant only for H2461 and JMN-1B cell lines ($P = 0.0007$ and 0.0169 , respectively). **B**, mesothelioma cell lines grown in the presence of 1 μ g/mL anti-HGF-neutralizing antibody (anti-HGF Ab) for 5 days. $*$, $P = 0.047$, differences between treatment and control is only significant for H2461. **C**, H2461 cell lines stably expressing Met shRNA were generated. Two clones were analyzed further and both showed significant knockdown of Met and a decrease in p-Akt (*inset*). The growth of both clones is significantly retarded compared with the parental cell line [$P = 0.0001$ (clone 6) and 0.0006 (clone 19)]. **D**, a doxycycline-inducible Met shRNA system was generated. With the addition of 5 μ g/mL doxycycline (*Dox*) for 72 hours, there was complete Met knockdown (*inset*). Some baseline Met knockdown is also observed in the absence of doxycycline. The growth in the presence of doxycycline is significantly retarded compared with the parental cell line ($P = 0.0022$), the cell line expressing the repressor alone ($P = 0.0003$), or the same cell line in the absence of doxycycline ($P = 0.0083$).

cycle arrest in H2461 and JMN-1B. There was no correlation between the presence or degree of baseline phosphorylation of Met receptor and the effect of PHA-665752 on cell growth of the cell lines examined. We chose 1 μ mol/L PHA-665752 for subsequent experiments because this concentration was near the IC_{50} for H2461 and JMN-1B and because, at greater concentrations, no additional effects on G_1 -S arrest or inhibition of Met phosphorylation were observed.

Growth inhibition by anti-hepatocyte growth factor-neutralizing antibody and by Met interference RNA. To further investigate the role of the HGF autocrine loop in cell growth, we next treated the cells with anti-HGF-neutralizing antibody. In H2461 cells, the anti-HGF-neutralizing antibody significantly inhibited growth of MPM cells, although the magnitude of inhibition was modest (Fig. 4B). In the other three MPM cell lines, there was no significant growth inhibition by the anti-HGF-neutralizing antibody (Fig. 4B). As an independent method of disrupting the Met receptor signaling, we used RNAi to down-regulate Met expression in H2461 and JMN-1B cell lines. Of the three stable shRNA constructs examined, Met 345 was the most effective at causing knockdown of Met receptor expression with virtual complete knockdown as shown by Western blotting (Fig. 4C; data not shown). H2461 cells transfected with the Met 345 exhibited significantly slower growth rates compared with parental cells or cells expressing vector alone (Fig. 4C). Similar findings were observed for JMN-1B (data not shown). In H2461, phosphorylation of Akt was also substantially reduced similar to cells treated with PHA-665752 (Fig. 3). To mimic the short-term effects of PHA-665752 treatment, we constructed a doxycycline-inducible shRNA against the Met receptor. As can be seen in Fig. 4D, growth of RNAi-expressing cells in the absence of doxycycline is reduced. This discrepancy is likely because the inducible system is leaky as shown by reduced Met expression. However, with the

addition of doxycycline, there is complete inhibition of Met expression and a significant effect on growth in H2461. Similar findings were observed for JMN-1B (data not shown). Taken together, our RNAi studies confirm the findings observed with PHA-665752 and show that Met inhibition can decrease the growth of MPM cell lines *in vitro*.

Cell morphology. HGF stimulation of the Met receptor causes cell-cell dissociation or cell scattering in many different cell types (44). We examined the effects of PHA-665752 on MPM cell morphology especially from the aspect of cell-cell contact, as previous studies have shown that PHA-665752 can inhibit cell scattering by HGF (36). As shown in Fig. 5A, exposure to PHA-665752 resulted in increased cell-cell adhesion of JMN-1B cells, a cell line exhibiting typical sarcomatoid morphology. In contrast, no significant morphologic changes were observed in H2373, another sarcomatoid MPM cell (Fig. 5B), or in the epithelial MPM (H2461 and H2591) cells with PHA-665752. Similar although less dramatic effects were observed only in JMN-1B following treatment with the HGF-neutralizing antibody (data not shown).

Cell migration. To test the effect of PHA-665752 on cell motility, we did scratch wound migration assay. Of four MPM cells, JMN-1B had the highest (>500 μ m), H2461 (193 ± 61 μ m) and H2373 (124 ± 48 μ m) had intermediate, and H2591 (50 ± 18 μ m) had the lowest migrating distance per 24 hours (Fig. 6A; data not shown). The treatment with PHA-665752 significantly reduced the rate of migration in H2461 cells (Fig. 6A). The similar finding was observed when H2461 cells were treated with HGF-neutralizing antibody (Fig. 6A). In contrast, there was no significant change in rate of migration in the other three MPM cells by the treatment with PHA-665752 or anti-HGF-neutralizing antibody (Fig. 6A; data not shown). We next examined the effects of Met 345 shRNA on H2461 cell

migration. H2461 cells with Met shRNAs clone 6 (complete knockdown) and clone 19 (partial knockdown) also exhibited a lower rate of migration than parental or vector alone cells (Fig. 6B). These results suggest that inhibition of the HGF/Met pathway either through inhibition of the ligand or the receptor can affect MPM cell migration.

Cell invasion. HGF/Met signaling has been shown to promote cell invasion in various cancer cells, including MPM (33). Because migration is considered an important step of HGF-induced invasion, and HGF-induced cell dissociation is believed to correlate with higher invasive potential (45, 46), we hypothesized that PHA-665752 could inhibit cell invasion of MPM cells. We tested the effect of PHA-665752 on cell invasion using a Matrigel cell invasion assay. As shown in Fig. 7, the amount of cell invasion in the MPM cell lines corresponds to their motility observed in the migration assay (Fig. 6; data not shown). In H2461 and JMN-1B cells, PHA-665752 significantly retarded the rate of invasion (Fig. 7A). In contrast, in H2373 or H2591, the rate of invasion was not affected by PHA-665752 treatment (Fig. 7B).

Discussion

MPM is an aggressive malignancy with no known curative therapeutic modality and is associated with a poor patient prognosis. Although there have been advances in the use of chemotherapy in this disease, most patients die of their disease within a year of diagnosis (5). This has prompted the identification and validation of other effective therapeutic targets. The Met receptor has diverse roles in tumorigenesis, including cell growth, motility, invasion, metastasis, and angiogenesis (16–18). Mesotheliomas commonly express both Met and HGF and this expression is linked to the pathogenesis of the disease (6, 8, 34, 35). In the present study, we examined *in vitro* effects of inhibiting the Met receptor using both a

specific Met tyrosine kinase inhibitor (PHA-665752) and interference RNA technology.

Our studies indicate that, despite Met expression in 100% of MPM cell lines, only 2 of the 10 (20%) cell lines undergo cell cycle arrest at the G₁-S boundary and growth inhibition following the addition of PHA-665752. In these two cell lines, H2461 and JMN-1B, we detect autocrine HGF production. Our findings suggest that the presence of HGF/Met autocrine loop results in dependency on Met signaling in these cell lines. This is further highlighted by the fact that in cell lines without HGF production, H2373 and H2591, although there is basal phosphorylation of the Met receptor and despite its inhibition by PHA-665752 there is no inhibition of growth or of the downstream signaling pathways (Figs. 3 and 4). Using RNAi as an independent method to inhibit the Met receptor, we are able to show growth inhibition of H2461 and JMN-1B cell lines. Treatment of H2461 with an anti-HGF antibody also inhibited cell growth and migration consistent with dependence of HGF/Met signaling axis in this cell line. However, no effects were observed in JMN-1B cells with the anti-HGF antibody (Fig. 4B) despite growth inhibition by PHA-665752 in this cell line (Fig. 4A). This observation is somewhat surprising given that JMN-1B had the highest detectable amount of HGF secretion. Thus, although the autocrine production of HGF may be an indicator of dependency for HGF/Met for growth and its inhibition by PHA-665752, the absolute amount produced, as detected by ELISA, may not be a direct reflection of the magnitude of growth inhibition observed by anti-HGF antibodies. These findings also suggest that, in tumors with autocrine HGF production, inhibition of Met using a tyrosine kinase inhibitor, such as PHA-665752, may be a more effective therapeutic strategy than treatment with antibodies directed at HGF.

We show that PHA-665752 exhibits a cytostatic rather than a cytotoxic effect on MPM cell lines as shown by induction of G₁-S arrest and the lack of a sub-G₁ fraction, which would be consistent with apoptosis. Christensen et al. (36) reported that PHA-665752 treatment of GTL-16, a gastric cancer cell line, induced significant apoptosis; thus, our observations may be cell specific.

Met receptor mutations leading to an increase in receptor tyrosine kinase activity have been described previously in hereditary papillary renal cell carcinomas, hepatocellular carcinomas, and head and neck squamous cell carcinomas (23, 25, 26, 47). Prior studies have also shown that some but not all mutations in the Met tyrosine kinase confer an increase in sensitivity to the Met kinase inhibitor SU011274 (48). Similar findings have been observed in other malignancies, such as non-small cell lung cancer, where cell lines harboring mutations in the EGFR are more sensitive to the EGFR tyrosine kinase inhibitor gefitinib than those with wild-type EGFR (49). We thus investigated our 10 mesothelioma cell lines for mutations in the Met receptor but did not detect any mutations.

We next examined the effects of PHA-665752 on Met receptor signaling in two sensitive and two resistant MPM cell lines. In the two sensitive cell lines, H2461 and JMN-1B, and not in the two resistant MPM cells, PHA-665752 caused the decrease in phosphorylation of p70S6K and/or extracellular signal-regulated kinase 1/2 and Akt and an increase in p27 (Fig. 3); consistent with prior studies using Met tyrosine kinase inhibitors but unlike prior studies, all of our analyses are done

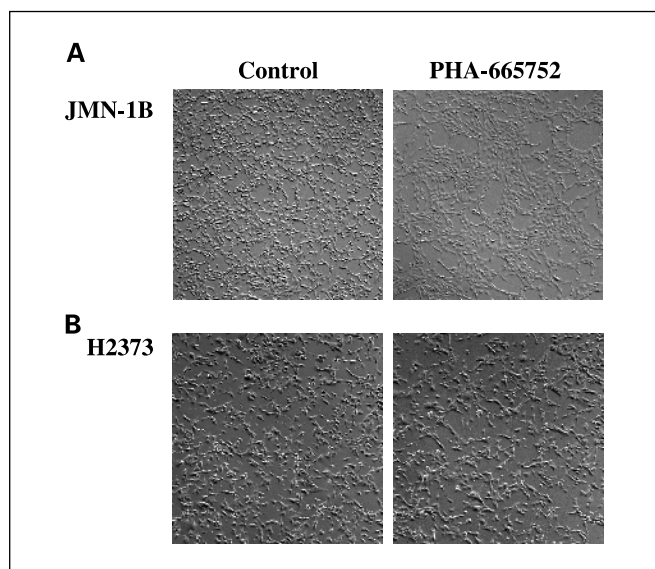


Fig. 5. Met inhibition alters the morphology of JMN-1B mesothelioma cells. JMN-1B (A) or H2373 (B) cells were treated with or without 1 μ mol/L PHA-665752 and photographed after 24 hours. Treatment with PHA-665752 induced a more clustered appearance of JMN-1B, whereas H2373 is unaltered.

in full serum-containing medium in the absence of HGF stimulation (28, 36, 48). Although the effects on cell cycle distribution are similar for H2461 and JMN-1B following PHA-665752 treatment (Fig. 2), the effects on p70S6K and p27 in JMN-1B are less striking than in H2461 (Fig. 3), suggesting that additional pathways may be involved in mediating cell cycle arrest in this cell line. Although the phosphorylation of Met was inhibited in the resistant H2373 and H2591 cell lines, this was not coupled to inhibition of any downstream signaling pathways (Fig. 3). We have observed previously similar findings for the dual EGFR/ErbB2 inhibitor lapatinib in mesothelioma cell lines (11). In those studies, phosphorylation of EGFR was inhibited in all MPM cell lines tested but, coupled with the inhibition of downstream signaling pathways, only in those cell lines that were also growth inhibited by lapatinib. These findings have therapeutic implications in the design of future clinical studies using Met inhibitors. Because both sensitive and resistant cell lines all express both total and phosphorylated forms of Met, choosing patients for clinical trials based on Met expression alone may not be predictive of a subsequent clinical response to Met inhibitors. Alternatively choosing patients based on a mechanism of Met activation (such as presence of an autocrine loop or activating mutation) may be a more effective method of identifying

tumors that are dependent on Met signaling for their growth. Our *in vitro* studies do not address the possibility of paracrine activation of the Met receptor in patients with MPM. This likely occurs in some mesotheliomas, as HGF staining has also been observed in stromal fibroblasts and tumor-associated macrophages by immunohistochemistry in MPM tumors where HGF is also expressed on the MPM cells themselves (8, 50). Thus, the therapeutic implications from our findings may be applicable to an even larger portion of patients with mesothelioma.

HGF activation of the Met receptor is known to induce morphologic cell changes and cell scattering in various malignant cell types, including mesotheliomas (14, 15, 32). We observed that PHA-665752 induced a morphologic transformation of the sarcomatoid JMN-1B cell line resulting in increased cell-cell contact consistent with inhibition of HGF-induced cell scattering in this cell line (Fig. 5). However, we did not observe this in epithelial H2461 cell line, which also has autocrine HGF production; thus, these observations may be mesothelioma subtype specific. The effects on cell migration were also different in these two cell lines. Disruption of Met signaling pathway with either PHA-665752, anti-HGF antibodies, or Met shRNA all significantly decreased migration of H2461, whereas this was unaltered in JMN-1B (Fig. 6; data not

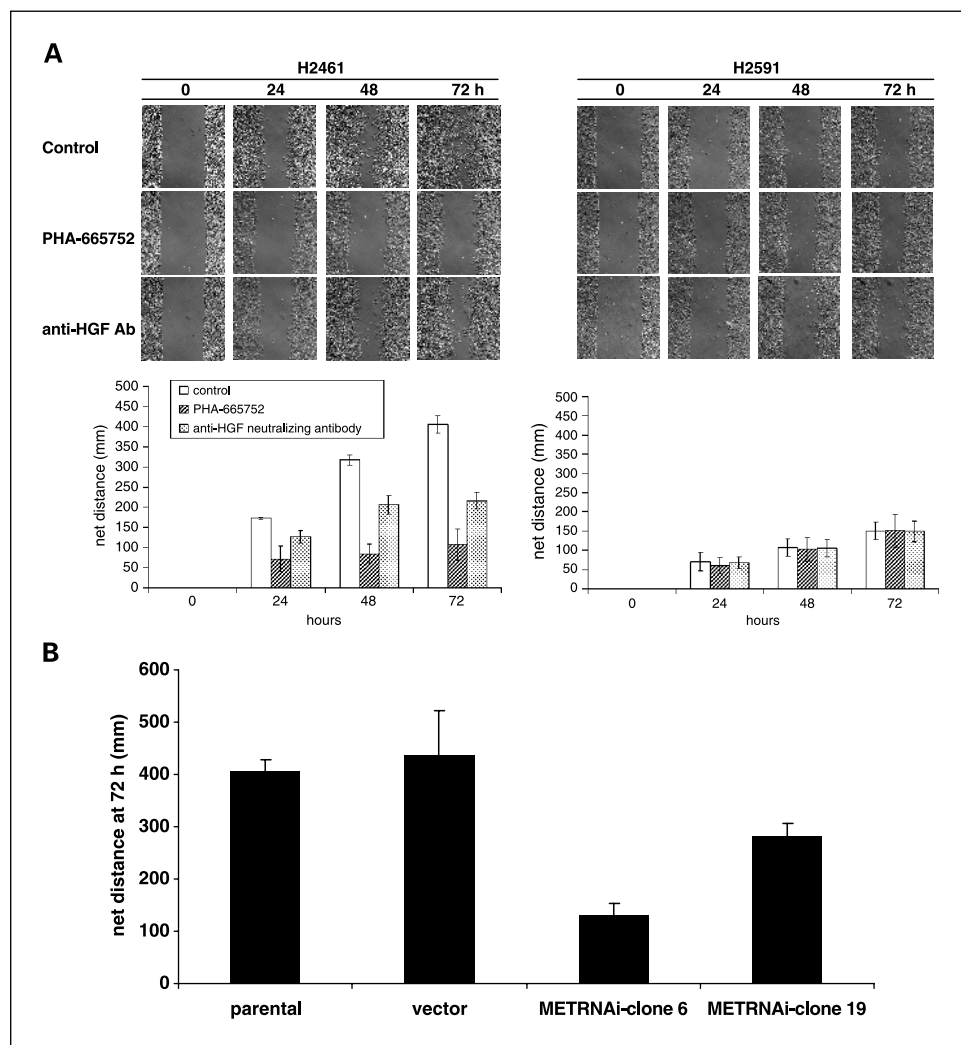


Fig. 6. Met inhibition alters migration of H2461. **A**, H2461 and H2591 cell lines were grown to confluence and treated with either 1 $\mu\text{mol/L}$ PHA-665752 or 1 $\mu\text{g/mL}$ anti-HGF antibody before an *in vitro* scratch assay. The scratched region was photographed at the indicated times and the migration distance was measured at three fixed positions (*top*, *middle*, and *bottom*). The migration is significantly slower in H2461 cell lines treated with either PHA-665752 or the anti-HGF antibody at all time points ($P < 0.05$). **B**, migration was assayed at 72 hours in H2461 cells expressing stable Met shRNAs. The migration of the two H2461 clones with stable expression of the Met shRNAs is significantly slower [$P = 0.0001$ (clone 6) and 0.0035 (clone 19)] compared with the parental cell line.

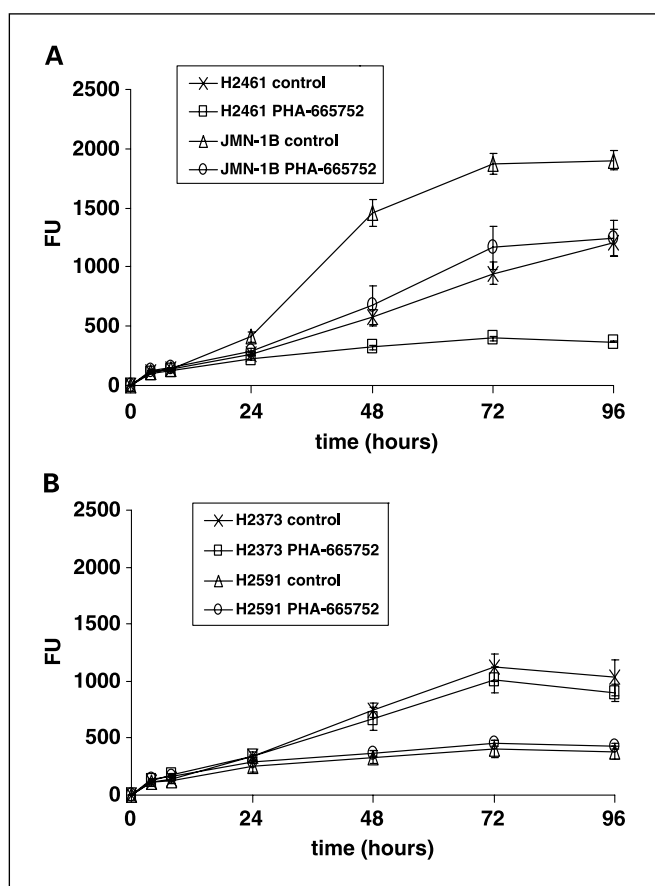


Fig. 7. PHA-665752 reduces mesothelioma cell invasion through Matrigel. **A.** invasion of H2461 and JMN-1B was assessed over 96 hours. In cells treated with PHA-665752, invasion was significantly slowed after all time points beyond 8 hours for JMN-1B ($P < 0.05$) and at 48, 72, and 96 hours for H2461 ($P < 0.05$). **B.** invasion of H2373 and H2591 was assessed over 96 hours. There was no effect of PHA-665752 treatment on cell invasion.

shown). However, PHA-665752 significantly inhibited the invasion through Matrigel of both H2461 and JMN-1B, although the effects were more pronounced for H2461 (Fig. 7). The effects of inhibiting Met activation in MPM cells, decreased cell scattering, migration, and invasion, are as expected, opposite of those observed previously with HGF

stimulation of MPM cell lines (32, 33). Thus, in addition to the effect on cell growth, these findings suggest another therapeutically important role for Met inhibition, because one of the clinical characteristics of MPM is local invasion into adjacent structures, such as the chest wall or diaphragm. Thus, inhibiting these processes may provide significant clinical benefits for patients with MPM. Molecular mechanisms, by which HGF/Met signaling accelerates cell scattering, motility, or invasion, are thought to involve activation of FAK and paxillin (51–53). Despite the effects of PHA-665752 on motility and invasion in H2461 and JMN-1B cells, we observe no decrease in the phosphorylation of FAK or paxillin (Fig. 3; data not shown). In fact, we observe a slight increase in phosphorylation of FAK (Tyr³⁹⁷ and Tyr⁸⁶¹) and paxillin (Tyr³¹ and Tyr¹¹⁸) in the H2461 cell line (Fig. 3; data not shown). Our findings are in contrast to those of Christensen et al. (36) who showed recently that PHA-665752 inhibited migration and FAK phosphorylation (pY861) in both NCI-H441 (non-small cell lung cancer) and GTL-16 (gastric cancer). However, in their studies, FAK phosphorylation is assayed at either 10 or 30 minutes after exogenous HGF stimulation to serum-starved cells with or without PHA-665752, whereas we used MPM cells grown in serum-containing medium without additional HGF and assayed the effects after 24 hours of treatment. These differences between our findings are presently being investigated.

Taken together, our study provides evidence supporting the possibility of therapeutic targeting HGF/Met signaling in MPM. Disrupting HGF/Met signaling is potentially attractive as it may not only inhibit MPM growth but also alter the rate of migration and invasion in a disease that is clinically characterized by local extension. However, the effects of Met receptor inhibition are limited to a minority of MPM cell lines, those that also produce HGF. Our studies would predict that clinical trials of Met inhibitors, such as PHA-665752, in MPM would be most effective in patients whose tumors express both Met and HGF. Furthermore, PHA-665752 treatment may also be effective in other tumor types that exhibit autocrine HGF production, such as some non-small cell lung carcinomas (54).

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