A Selective Small Molecule Inhibitor of c-Met Kinase Inhibits c-Met-Dependent Phenotypes in Vitro and Exhibits Cytoreductive Antitumor Activity in vivo

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ABSTRACT

The c-Met receptor tyrosine kinase and its ligand, hepatocyte growth factor (HGF), have been implicated in the development and progression of several human cancers and are attractive targets for cancer therapy. PHA-665752 was identified as a small molecule, ATP-competitive, active-site inhibitor of the catalytic activity of c-Met kinase (K_i, 4 nM). PHA-665752 also exhibited >50-fold selectivity for c-Met compared with a panel of diverse tyrosine and serine-threonine kinases. In cellular studies, PHA-665752 potently inhibited HGF-stimulated and constitutive c-Met phosphorylation, as well as HGF and c-Met-driven phenotypes such as cell growth (proliferation and survival), cell motility, invasion, and/or morphology of a variety of tumor cells. In addition, PHA-665752 inhibited HGF-stimulated or constitutive phosphorylation of mediators of downstream signal transduction of c-Met, including Gab-1, extracellular regulated kinase, Akt, signal transducer and activator of transcription 3, phospholipase C y, and focal adhesion kinase, in multiple tumor cell lines in a pattern correlating to the phenotypic response of a given tumor cell. In in vivo studies, a single dose of PHA-665752 inhibited c-Met phosphorylation in tumor xenografts for up to 12 h. Inhibition of c-Met phosphorylation was associated with dose-dependent tumor growth inhibition/growth delay over a repeated administration schedule at well-tolerated doses. Importantly, persistent, potent cytodestructive activity was demonstrated in a gastric carcinoma xenograft model. Collectively, these results demonstrate the feasibility of selectively targeting c-Met with ATP-competitive small-molecules and suggest the therapeutic potential of targeting c-Met in human cancers.

INTRODUCTION

RTKs regulate many key processes in mammalian development, cell function, and tissue homeostasis. These diverse processes include cell growth and survival, organ morphogenesis, neovascularization, and tissue repair and regeneration, among others. Dysregulation of RTKs by mutation, gene rearrangement, gene amplification, and overexpression of both receptor and ligand have been implicated as causative factors in the development and progression of numerous human cancers. The validity of RTKs as therapeutic targets is illustrated by the successes of Gleevec targeting bcr-abl in chronic myelogenous leukemia and c-Kit in gastrointestinal stromal tumors, Herceptin in HER-2 overexpressing breast cancers, and Iressa in non-small cell lung cancers (1).

The c-Met RTK is the only known high-affinity receptor for HGF, also known as scatter factor (2, 3). Binding of HGF to the c-Met extracellular ligand-binding domain results in receptor multimerization and phosphorylation of multiple tyrosine residues at the intracellular region. Tyrosine phosphorylation at the c-Met juxtamembrane, catalytic, and cytoplasmic tail domains regulates the internalization, catalytic activity, and docking of regulatory substrates, respectively (4–7). Activation of c-Met results in the binding and phosphorylation of adaptors such as Gab-1, Grb2, Shc, and c-Cbl, and subsequent activation of signal transducers such as PI3K, PLC-γ, STATs, ERK 1 and 2, and FAK (8).

c-Met and HGF are expressed in numerous tissues, and their expression is normally confined predominantly to cells of epithelial and mesenchymal origin, respectively (9, 10). The transduction of signaling and subsequent biological effects of HGF by c-Met has been shown to be important in epithelial-mesenchymal interaction and regulation of cell migration, invasion, cell proliferation and survival, angiogenesis, morphogenic differentiation, and organization of 3-dimensional tubular structures (e.g., renal tubular cells, gland formation, and so forth; Refs. 11, 12). In addition, c-Met and HGF are each required for normal mammalian development, and they are believed to be important in regulating epithelial-mesenchymal transitions during organ morphogenesis (13–15).

c-Met and HGF are dysregulated in human cancers and are also believed to contribute to dysregulation of cell growth, tumor cell dissemination, and tumor invasion during disease progression and metastasis. This suggests that c-Met and HGF may be attractive candidates for targeted cancer therapy. c-Met and HGF are highly expressed relative to surrounding tissue in numerous cancers, and their expression correlates with poor patient prognosis (12). c-Met activating point mutations in the kinase domain are implicated as the cause of hereditary papillary renal carcinoma (16). In addition, kinase domain mutations have been observed in sporadic papillary renal carcinoma, ovarian cancer, childhood hepatocellular carcinoma, and gastric cancer (16–18). Furthermore, mutations in head and neck squamous cell carcinoma metastases, and mutations and gene amplification in colorectal cancer metastases, implicate c-Met in the metastatic progression of these cancers (19, 20). Cell lines engineered to express high levels of c-Met and HGF (autocrine loop) or mutant c-Met displayed a proliferative, motogenic, and/or invasive phenotype, and grew as metastatic tumors in nude mice (21–25). In addition, transgenic mice overexpressing c-Met, HGF, or mutant c-Met display a tumorigenic and metastatic phenotype (26, 27).

c-Met receptor antagonists including ribozymes, dominant-negative proteins, and HGF kringle-variants such as NK4 have been shown to reverse c-Met/HGF biological phenotypes, and inhibit tumor growth and dissemination (28–31). Collectively, these data additionally support the potential for targeting c-Met with small-molecule therapeutics. Recently, K252a, a Staurosporine analogue and inhibitor of multiple protein kinases, demonstrated submicromolar c-Met activity, and modulated wild-type and mutant c-Met-dependent function and dissemination of tumor cells in vivo supporting the concept of development of small molecule c-Met RTK inhibitors (32).
To address the issue of compound selectivity in characterization of c-Met function, we have identified a series of selective small molecule c-Met RTK inhibitors. A prototype from this small-molecule family, PHA-665752, was shown to potently inhibit c-Met enzyme with a $K_i$ of 4 nM and an IC$_{50}$ of 9 nM. PHA-665752 was >50-fold selective over a large panel of other tyrosine and serine-threonine kinases, supporting its utility in selectively characterizing c-Met-dependent signal transduction, function, and mechanism-of-action in vivo. In cells, PHA-665752 also inhibited c-Met phosphorylation and c-Met-dependent motility, invasion, and proliferation with IC$_{50}$ values in the low nanomolar range, and modulated known c-Met signal transducers including ERKs, Akt, and FAK. In mouse tumor models, PHA-665752 inhibited Met phosphorylation and signal transduction, which correlated with tumor growth inhibition or tumor regression at well-tolerated doses. These results support the therapeutic potential of targeting c-Met in cancers where c-Met plays a role in tumor growth or metastasis.

**MATERIALS AND METHODS**

**Compound.** PHA-665752 (3Z)-5-[[2,6-dichlorobenzyl)sulfonyl]-3-[[3,5-dimethyl-4-[(2R)-2-(pyrrolidin-1-ylmethyl)pyrrolidin-1-yl]carbonyl]-1H-pyrrol-2-yl)methylene]-1,3-dihydro-2H-indol-2-one (Fig. 1) was synthesized at SUGEN, Inc.

Cells. NCI-H441 human lung carcinoma, A549 human lung carcinoma, BxPC-3 human pancreatic carcinoma, MDCK canine kidney epithelial, and RIE-1 cells were obtained from American Type Culture Collection (Rockville, MD), NIH3T3 or Chinese hamster ovary cells stably transfected with Ron or Flk-1 were generated at SUGEN. The MDCK cells used for motility assays were derived from a MDCK subclone with scattering properties. S114 cells, derived from NIH3T3 mouse fibroblasts engineered to stably express human c-Met and HGF, were provided by Dr. George Vande Woude (Van Andel Institute, Grand Rapids, MI). GTL-16 gastric carcinoma cells were originally derived from a MDCK subclone with scattering properties. S114 cells, provided by Dr. Michael Sadelain (SUGEN), were grown in 2% FBS in the presence of HGF (50 ng/ml) in tissue culture and passed weekly. S114 cells were transfected with Ron (300 ng/ml) and the resulting cells were selected with 2 µg/ml of G418 and maintained at 37°C in a humidified atmosphere at 5% CO$_2$. HGF was added at 10 ng/ml every 3 days. RTKs and other tyrosine kinases in PHA-665752 were measured as described previously (34).

**In Vitro Enzyme Assays.** The IC$_{50}$ values of PHA-665752 for the inhibition of c-Met and various other kinases were determined as described previously (34). Briefly, c-Met kinase domain GST-fusion protein was used for the c-Met assay, whereas recombinant human full-length or GST-kinase domain fusion proteins were used in other enzyme assays. IC$_{50}$ measurements of compound versus kinases were based on phosphorylation of kinase peptide substrates or poly-glu-tyr in the presence of ATP and divalent cation (MgCl$_2$, MnCl$_2$, 10–20 mM). The linear range (i.e., the time period over which the rate remained equivalent to the initial rate) was determined for each kinase, and all of the kinetic measurements and IC$_{50}$ determinations were performed within this range. $K_m$ values were calculated using the Eadie-Hofstee and Lineweaver-Burke methods with the final ATP concentrations within two to three times the $K_m$ value.

**Cell Assays**

**Cell Proliferation Assays.** S114, GTL-16, NCI-H441, or BxPC-3 cells were seeded in 96-well plates at 9000 cells/well in medium with 10% FBS. After incubation for 48 h in low serum (0.5% FBS, S114; 0.1% FBS, GTL-16, NCI-H441, and BxPC-3), cells were treated with different concentrations of PHA-665752 for 18 h at 37°C. HGF (50 ng/ml) was added for 18 h before BrdUrd for studies involving GTL-16, H441, and BxPC-3. After incubation with BrdUrd labeling reagent for 1 h (Sigma Biochemicals, St. Louis, MO), cells were fixed and BrdUrd incorporation into newly synthesized DNA was assessed using anti-BrdUrd peroxidase-conjugated antibody followed by colorimetric determination at 630 nm.

**Scatter Assay.** MDCK cells were seeded at low density (25 cells/well) in a 96-well plate in MEM with 10% FBS and grown until small colonies of 10–15 cells appeared. Cells were then stimulated with HGF/scatter factor (50 ng/ml) in the presence of various concentrations of PHA-665752 diluted in growth medium. After overnight incubation, colonies were fixed and stained with 0.2% crystal violet in 10% buffered formalin and assessed for scattering at each concentration visually and by an image analysis algorithm designed to measure scattering over several experimental replicates ($>$10).

**Migration Assay.** Cells were seeded in RPMI 1650 at 10% FBS, grown until confluent, and a gap was introduced by scraping cells with a P200 pipette tip. Cells were then stimulated to migrate across the gap with HGF (50 ng/ml) in the presence of various concentrations of PHA-665752 diluted in growth medium. After an overnight incubation, a qualitative assessment of inhibition of migration was performed at each concentration.

**Three-Dimensional Invasion/Tubulogenesis Assay.** Matrigel (Matrigel Basement Membrane Matrix; Becton Dickinson Labware) was thawed on ice. Matrigel was plated at 50 µl/well in 96-well plate and incubated for 20 min at 37°C to induce gel formation. RIE-1 cells were suspended at 2 x 10^5 cell/ml and mixed 1:1 with Matrigel on ice. We added 0.1 ml of the mixture per well. After 24 h, 0.1 ml DMEM ± 80 ng/ml HGF (R&D 294-9g-005) ± various concentrations of PHA-665752 were added on top of each Matrigel cell plug. Fresh medium ± HGF was added every 3 days. Photographs were taken after 6 days in culture.

**Apoptosis Assay.** Cells were seeded at 54,000 cells/well in 24-well plates in medium containing 2% FBS in presence and absence of HGF (50 ng/ml), and various concentrations of PHA-665752. After 72 h, medium was aspirated and replaced by a mixture containing ethidium bromide (200 nM) and acidine orange (200 nM; both Sigma Biochemicals, St. Louis, MO) in PBS (pH 7.4). Cells (1000/well) were counted under a microscope at ×20 with a fluorescent filter at 520 nm. Apoptotic cells stained brightly orange indicating presence of condensed chromatin and lack of nuclear membrane integrity. Necrotic cells stained a dull orange and were not included in this analysis. Results were expressed as percentage of apoptotic cells (1000 total cells counted).

**Soft Agar Assay.** Cells were seeded at 5000 cells/well in a 12-well plate in medium containing FBS (1%), in the presence and absence of HGF (100 ng/ml) and PHA-665752 over a base agar layer (0.5 ml of 1:1 mixture of 2% FBS containing medium and 1.2% agar solution). The plates were incubated (37°C, 5% CO$_2$), and the number and size of colonies were evaluated under each condition after 7–14 days.

**Antibodies.** Rabbit polyclonal antibodies included antihuman-met (sc-161; Santa Cruz Biotechnology Biotech, Santa Cruz, CA); antiphosphorylated-c-Met (pY1230/4/5; Biosource, Camarillo, CA); antiphosphorylated c-Met (pY1003; Biosource); anti-pY (SUGEN); anti-Gab-1 for immunoprecipitation; Santa Cruz Biotechnology, Inc.; antiphosphorylated-p44/42 ERK mitogen-activated protein kinase, (Thr 202/Tyr204; Cell Signaling Technology, Inc., Waltham, MA), antiphosphorylated-p44/42 ERK mitogen-activated protein kinase, (Thr 202/Tyr204; Cell Signaling Technology, Beverly, MA); anti-ERK mitogen-activated protein kinase, (Cell Signalling Technology); anti-phosphorylated-Akt (Ser473, IHC specific; Cell Signaling Technology); antiphosphorylated-Akt (Ser473, immunoblotting; Cell Signalling Technology, Beverly); antiphosphorylated-FAK Y861 (Biosource); anti-STAT-3 (Cell Signaling); antiphosphorylated STAT-3 (Th/705; Cell Signaling); anti-PLC-γ (Santa Cruz Biotechnology); antiphosphorylated PLC-γ.
Immunoblot Analysis. The expression and phosphorylation of c-Met and its signaling proteins were evaluated by immunoblotting with antibodies listed above. Briefly, protein lysates were made from pelleted cells or powdered frozen tissue as described previously (35) by incubation in lysis buffer with protease and phosphatase inhibitors [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM magnesium chloride, 10% Glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 0.5 mM sodium fluoride, apro tin (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), and phenylmethylsulfonyl fluoride (1 mM)] at 4°C. Protein lysates were cleared of cellular debris by centrifugation at 15,000 × g for 15 min, resolved by electrophoresis on 8% SDS-PAGE gels, and electrotransferred to a nitrocellulose membrane. In the experiments involving Gab-1 lysates were immunoprecipitated for the specific protein of interest before electrophoresis and transfer. Proteins were detected using standard immunoblotting procedures using the antibodies listed above.

Immunofluorescence Analysis. Cells used in immunofluorescence studies (GTL-16, NCI-H441, and MDCK) were grown on coverslips in six-well plates. The designated amounts of HGF and/or PHA-665752 were added to coverslips for each experiment. Briefly, cells were fixed in 100% methanol for 30 min at −20°C and washed four times in TBS (pH 7.4). Fixed cells were then: (a) blocked in 10% FBS in TBS for 60 min; (b) incubated in primary antibody (c-Met, sc-161; 1:1000) in 10% FBS in TBS for 60 min; (c) washed four times in TBS; (d) incubated with an antirabbit fluorescent-tagged secondary antibody (1:4000) in 10% FBS in TBS for 60 min; and (e) washed four times in TBS. Coverslips were then mounted on slides and evaluated by fluorescence microscopy using a Leica DMLB HC Photomicroscopy system.

In Vivo Studies

Animals. Female athymic mice (nu/nu, 8–12 weeks old) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed on a 12-h light/dark cycle with food and water provided ad libitum in a barrier facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Animals were examined before the initiation of experiments to ensure that they were healthy and acclimated to the laboratory environment. All of the procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with SUGEN Animal Care and Use Committee guidelines.

Target Modulation Studies

Tumor Pharmacodynamics. In vivo target modulation studies were performed in mice bearing S114 or GTL-16 tumor xenografts to determine the effect of PHA-665752 on c-Met phosphorylation in both single-dose and repeat-dose studies. For all of the target modulation studies, mice bearing established tumors (200–1200 mm3) were administered PHA-665752 or vehicle [l-lactate (pH 4.8) and 10% polyethylene glycol] via bolus i.v. tail vein injection at the desired dose in a volume of 150 μl. At the indicated times after administration, a blood sample was isolated from cardiac left ventricle using a syringe primed with heparin sulfate, mice were euthanized, and their tumors were resected. Resected tumors were immediately frozen and pulverized using a liquid nitrogen-cooled cryomortar and pestle. Tumor powders were processed into lysates by homogenization in cold lysis buffer with protease and phosphatase inhibitors (35). The amount of phosphorylated and total c-Met in each sample was determined by immunoblot analysis of protein lysates as described above.

Efficacy Studies

Repeated-Dose Administration Tumor Growth Inhibition. Efficacy studies were performed in athymic mice bearing S114 or GTL-16 tumor xenografts to determine the effect of PHA-665752 on tumor growth. Tumor cells (5 × 106 cells/animal) were implanted s.c. into the hindflank region of mice on day 0. Daily treatment with inhibitor was initiated when tumors were ∼50 mm3 or 400 mm3 in size for S114 and GTL-16, respectively. PHA-665752 or vehicle (described above) was administered as daily i.v. bolus via tail vein injection for up to 10 days. Tumors were measured twice weekly using Vernier calipers, and tumor volumes were calculated as the product of length × width × height. At the end of the study, tumor growth inhibition values were expressed as: 1 - (mean treated tumor mass/mean control tumor mass × 100). For the GTL-16 efficacy study a representative cohort (n = 3) of animals were sacrificed at the times indicated, their tumors resected, and a blood sample taken from the cardiac left ventricle using a syringe primed with heparin sulfate. Resected tumors were cut in half. One half was fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned for immunohistochemistry, and the other half immediately frozen and subsequently processed into tumor powder, and ultimately protein lysates, for Western blot analysis as described above.

Immunohistochemical Studies. Resected mouse xenografts were fixed in 10% neutral buffered formalin (Protocol; Fisher Scientific, Pittsburgh, PA) with protease inhibitor (0.5 mM sodium fluoride) and phosphatase inhibitor (1 mM sodium orthovanadate). Tissue was fixed overnight and stored in 70% ethanol until embedded in paraffin. Four μm sections were cut and baked on Superfrost plus microscope slides (Fisher Scientific). The Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) was used according to manufacturers instructions for deparaffinization, antigen retrieval [citrate buffer (pH 7.4) at 94°C for 30 min] and immunohistochemical staining. H&E stains were performed for each section to gain orientation. Primary rabbit polyclonal antibodies used for immunohistochemical studies (c-Met, phosphorylated c-Met, and phospho-ERK, Akt, and FAK antibodies) are described above. The secondary antibody in each case was biotinylated goat anti-rabbit IgG (Vector). Stained sections were analyzed and documented using a Leica DMLB HC Photomicroscopy system.

RESULTS

Identification of PHA-665752 as a Potent and Selective Inhibitor of c-Met Activity and Function. Putative c-Met inhibitors were identified using biochemical assays with isolated GST-Met kinase domain and HGF-dependent cellular kinase, proliferation, and motility assays. In biochemical assays, PHA-665752 (structure in Fig. 1) demonstrated potent, ATP-competitive inhibition of c-Met kinase activity with an KI of 4 nM and IC_{50} of 9 nM. In addition to the potent biochemical activity against c-Met enzyme, PHA-665752 also demonstrated potent inhibition of c-Met RTK phosphorylation in NIH3T3 cells engineered to express high levels of c-Met and HGF (S114 cells), HGF-stimulated A549 lung carcinoma cells, and mouse B16-F1 melanoma cells (Table 1; Fig. 2A).

PHA-665752 was also evaluated for its activity against a panel of enzymes representing diverse families of tyrosine and serine-threonine kinases to determine its selectivity for c-Met relative to other kinases. In this evaluation, PHA-665752 exhibited >50-fold selectivity for c-Met enzyme compared with the majority of kinases evaluated (Table 2). The exceptions were the Ron and VEGFR2 RTKs, against which PHA-665752 exhibited IC_{50} values of 68 and 200 nM, respectively, compared with a 9 nM IC_{50} versus c-Met kinase. In contrast,

Table 1 Summary of cellular activity of PHA-665752

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Cell line</th>
<th>Mean IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophosphorylation</td>
<td>S114</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>B16F1</td>
<td>0.05</td>
</tr>
<tr>
<td>Motility</td>
<td>MDCK</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>H441</td>
<td>0.05</td>
</tr>
<tr>
<td>Proliferation</td>
<td>S114</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>184B5</td>
<td>0.018</td>
</tr>
</tbody>
</table>

* Autophosphorylation IC_{50} in S114 determined using cell-based ELISA.
* Autophosphorylation IC_{50} in A549 and B16F1 estimated based on Visual immunoblotting assessment.
* An "*" denotes estimated value.
* Motility IC_{50} in MDCK determined based on scattering algorithm and image analysis over several drug concentrations and replicates.
* Motility IC_{50} in NCI-441 estimated based on Visual assessment of migration several drug concentrations and replicates.
* Proliferation IC_{50} in based on quantitation of BrDU incorporation.

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>20-fold cellular selectivity for c-Met versus Ron kinase was demonstrated by evaluating PHA-665752 for its ability to inhibit Ron autophosphorylation in Chinese hamster ovary cells engineered to express Ron. Similarly, >50-fold cellular selectivity versus VEGFR2 was demonstrated by evaluating PHA-665752 for its ability to inhibit VEGF-stimulated VEGFR2 phosphorylation in NIH3T3 cells engineered to express VEGFR2 (Table 2). These data indicate selectivity at concentrations used in cellular mechanistic studies and at levels of compound achieved in vivo (Table 2). Collectively, these data demonstrate that PHA-665752 is a potent and selective tool for the evaluation of c-Met-dependent cellular functions, signal transduction, and potentially its role in tumorigenesis.

Inhibition of c-Met kinase activity by PHA-665752 also correlated closely with inhibition of HGF- and c-Met-dependent functions such as motility, invasion, and growth regulation. In the characterization of the role of Met and HGF in cell function, HGF has been demonstrated to cause: (a) epithelial-derived cells grown in tight islands to undergo cell dissociation and scattering; (b) migration of cells across interstices in confluent cultures; (c) invasion and branching tubulogenesis of epithelial and tumor cell lines; and (d) mitogenic properties in primary epithelial cells (e.g., breast, hepatocytes, and lung) and certain tumor cell types (36, 37). Consistent with these phenomena, PHA-665752 potently inhibited: (a) HGF-dependent cell scattering of MDCK canine kidney epithelial cells; (b) HGF-dependent cell migration in NCI-H441 lung carcinoma cells; (c) invasion and branching morphogenesis of RIE cells; and (d) HGF-dependent cell proliferation (BrdUrd incorporation) in 184B5 mammary epithelial cells and S114 cells with IC50 values in the same range as those observed in kinase assays (Table 1; Fig. 2, B, C, and E). In contrast, PHA-665752 had no effect on cell viability in non-Met-expressing NIH3T3 mouse fibroblasts at concentrations up to 18 μM.

Characterization of the Effect of PHA-665752 on Signal Transduction in Tumor Cells and Correlation with c-Met-Dependent Function. PHA-665752 was also evaluated for its effect on phosphorylation of proteins known to transduce c-Met-dependent signals in cell lines that exhibit c-Met-dependent functional properties. In each of these cell lines, PHA-665752 was evaluated for its effect on constitutive or HGF-stimulated cell morphology, cell growth, and

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Fig. 2. Effects of PHA-665752 on (A) HGF-induced c-Met phosphorylation in A549 cells, (B) HGF-induced motility in MDCK cells, (C) HGF-induced migration in NCI-H441 cells, (D) morphology of GTL-16 cells, and (E) invasion/tubulogenesis of RIE cells. A. A549 cells were exposed to the indicated concentrations of PHA-665752 for 4 h. Cells were then stimulated with HGF (50 ng/ml), denoted by (+) for 10 min. c-Met protein expression and phosphorylation state were determined after immunoprecipitation of the receptor from tumor lysate and subsequent immunoblot analysis as described in “Materials and Methods.” B, MDCK cells were grown as small colonies at low density and treated with HGF (50 ng/ml) in the presence of various concentrations of PHA-665752. After an overnight incubation, cells in absence or presence of HGF or PHA-665752 (0.1 μM) were visualized via c-Met immunofluorescence staining and microscopy. C, NCI-H441 cells were grown to high density and a gap was introduced with a P200 pipette tip. Cells were then stimulated to migrate across the gap with HGF (50 ng/ml) in the presence of various concentrations of PHA-665752. After an overnight incubation, cells in absence or presence of HGF or PHA-665752 (0.1 μM) were visualized via c-Met immunofluorescence staining and microscopy. D, GTL-16 cells were cultured overnight in the presence and absence of HGF (50 ng/ml) and PHA-665752 (0.1 μM), visualized by light microscopy, and photographed. E, RIE cells were added to Matrigel and grew as small plugs. After 24 h, cells were stimulated with HGF (80 ng/ml) to invade and form tube-like structures in Matrigel in the presence or absence of various concentrations of PHA-665752.
Table 2: PHA-665752 activity against c-Met compared with other kinases

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Kinase Family and Group</th>
<th>Kinase Assay IC₅₀ (µM)</th>
<th>Cellular Assay IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Met</td>
<td>Class X RTK</td>
<td>0.009</td>
<td>0.042</td>
</tr>
<tr>
<td>Ron</td>
<td>Class X RTK</td>
<td>0.088</td>
<td>0.9</td>
</tr>
<tr>
<td>Flik</td>
<td>Class X RTK</td>
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<td>2.5</td>
</tr>
<tr>
<td>abl</td>
<td>abl TK</td>
<td>1.4</td>
<td>ND²</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Class IV RTK</td>
<td>3</td>
<td>ND³</td>
</tr>
<tr>
<td>EGF</td>
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<td>ND³</td>
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<td>c-src</td>
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<td>ND³</td>
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<tr>
<td>IGF1R</td>
<td>Class II RTK</td>
<td>&gt;10</td>
<td>ND³</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Class III RTK</td>
<td>&gt;10</td>
<td>ND³</td>
</tr>
<tr>
<td>AURORA2</td>
<td>AUR TK</td>
<td>ND³</td>
<td>ND³</td>
</tr>
<tr>
<td>PKA, PKBα</td>
<td>AGC STK</td>
<td>ND³</td>
<td>ND³</td>
</tr>
<tr>
<td>cdk 2, p38α</td>
<td>CMGC STK</td>
<td>ND³</td>
<td>ND³</td>
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<tr>
<td>MK2,3</td>
<td>CAMK STK</td>
<td>ND³</td>
<td>ND³</td>
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</tbody>
</table>

* Family and group names as follows: RTK, receptor tyrosine kinase, TK, tyrosine kinase; STK, serine-threonine kinase; AGC contains PKA, PKB, PKC families, CMGC contains CDK, MAPK, GSK3; and CLK families, CAMK, Calcium/calmodulin-dependent protein kinases.
* Kinase IC₅₀ values based on quantitation of substrate phosphorylation by recombinant enzyme.
* Cellular IC₅₀ values estimated based on visual assessment of immunoblots of receptor tyrosine phosphorylation in cell lines engineered to express receptor of interest.
* ND, not determined.

motogenic properties in monolayer as well as anchorage-independent growth in soft agar (summarized in Table 3). This analysis of c-Met-dependent signal transduction and functional response is focused on concentrations of PHA-665752 (~0.1 µM in monolayer, 0.2 µM in agarose) that demonstrated: (a) complete inhibition of c-Met phosphorylation (in monolayer and colonies in agarose); and (b) selectivity for c-Met versus other kinases evaluated. PHA-665752 did not demonstrate an effect on cell growth or viability in c-Met-negative control cells at 10-fold above the concentrations at which the analyses were performed (data not shown). [Negative control cells included cells in which inhibition of c-Met is predicted to have minimal consequences (e.g., NIH3T3-VEGFR2, rat-1-N-myc fibroblasts, and SK-BR-3 breast)].

The cell lines evaluated included GTL-16 gastric carcinoma, NCI-H441 lung carcinoma, and BxPC-3 pancreatic carcinoma. GTL-16 is derived from a poorly differentiated gastric adenocarcinoma, which contains a c-Met gene locus amplified multiple times, resulting in expression of high levels of constitutively active c-Met. In GTL-16 cells, PHA-665752 completely inhibited growth in soft agar, inhibited cell proliferation, and induced apoptosis in both the presence and absence of HGF at concentrations that inhibited tyrosine phosphorylation of c-Met (Fig. 3, A–C). Furthermore, treatment of GTL-16 gastric carcinoma cells with PHA-665752 caused reversion from a dedifferentiated, rounded morphology to a differentiated, flat morphology and resulted in increased cell-cell contact (Fig. 2D). PHA-665752 had no effect on GTL-16 cell migration in the presence or absence of HGF, NCI-H441 is derived from a papillary lung adenocarcinoma, expresses high levels of c-Met, and demonstrates constitutive phosphorylation of the c-Met RTK. In NCI-H441 cells, PHA-665752 inhibited growth in soft agar in both the presence and absence of HGF, inhibited constitutive and HGF-stimulated cell proliferation, and inhibited HGF-stimulated migration at low nanomolar concentrations of PHA-665752 (Fig. 2C; Fig. 3, A–C). PHA-665752 had no effect on cell morphology in NCI-H441 cells. BxPC-3 is derived from a pancreatic adenocarcinoma and expresses moderate levels of c-Met. In BxPC-3 cells, HGF induced only a weak mitogenic response in monolayer and did not induce growth in soft agar, migration, or morphogenesis. The weak HGF-dependent mitogenic response was inhibited at low nanomolar concentrations of PHA-665752 (Fig. 3A). In contrast, PHA-665752 had no effect on any of the cell growth or proliferation endpoints in any of the c-Met negative control cell lines at the concentrations tested (i.e., NIH3T3-VEGFR2, rat-1-N-myc, and SK-BR-3).

As shown in Fig. 4, the effect of PHA-665752 on c-Met signal transduction was also characterized in each of these cell lines. In each cell line evaluated, constitutive or HGF-stimulated tyrosine phosphorylation at the c-Met kinase domain (i.e., total pY, pY1230/34, pY1349, and pY1003) was completely inhibited by PHA-665752 (0.1 µM). Also shown in Fig. 4, PHA-665752 potently inhibited total tyrosine phosphorylation of Gab-1, a c-Met docking protein that contributes to Met-dependent signaling through PI3K, PLC-γ, and Crk in each cell line evaluated. In contrast, downstream signal transduction proteins such as ERK, Akt, FAK, and STAT family members that regulate c-Met-dependent functions were affected in a cell-type-specific manner by PHA-665752.

In GTL-16 cells, in which c-Met demonstrated a role in regulation of cell growth and morphology, PHA-665752 potently inhibited constitutive signaling through ERK, Akt, FAK, PLC-γ, and STAT pathways (Fig. 4). The constitutive phosphorylation of these signaling proteins in the absence of HGF together with inhibition of these pathways by PHA-665752, a selective c-Met inhibitor, indicate that these pathways are dependent on the constitutive activity of c-Met in this cell line. In addition, the down-regulation of multiple signaling pathways by PHA-665752 in GTL-16 is consistent with the pleiotropic effect of c-Met on the regulation of multiple cellular functions.

Similarly, in H441 cells, in which c-Met demonstrated a role in cell growth and motility, PHA-665752 potently inhibited constitutive signaling through the ERK, Akt, and FAK pathways, and partially inhibited PLC-γ phosphorylation (Fig. 4). Similar to observations in GTL-16 cells, signaling molecules appeared to exhibit constitutive activity; however, addition of HGF did appear to additionally stimulate FAK phosphorylation (Fig. 4). The ability of HGF to additionally induce c-Src-dependent FAK phosphorylation and cell motility in NCI-H441 is consistent with reports that this pathway is necessary for motility (38, 39). In addition, the down-regulation of ERK, Akt, and FAK pathways by PHA-665752 is consistent with regulation of both cell growth and motility by c-Met in H441 cells. In contrast to GTL-16 and H441 cells, the only signaling molecules that were markedly modulated by HGF or PHA-665752 in BxPC-3 cells were

Table 3: Summary of PHA-665752 effect on cell function and signaling

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Colony growth (Soft-agar)</th>
<th>Cell proliferation (BrdUrd)</th>
<th>Cell survival (apoptosis)</th>
<th>Cell migration (scratch)</th>
<th>Cell morphology</th>
<th>Effect of PHA-665752 on signaling molecules²</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTL-16</td>
<td>Inhibited¹</td>
<td>Inhibited</td>
<td>Induced apoptosis²</td>
<td>No effect</td>
<td>Differentiation³</td>
<td>Erk 1/2, Akt, FAK, PLC-γ, STAT-3</td>
</tr>
<tr>
<td>NCI-H441</td>
<td>Inhibited</td>
<td>Inhibited</td>
<td>No effect</td>
<td>Inhibited</td>
<td>No effect</td>
<td>Erk 1/2, Akt, FAK</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>No colonies</td>
<td>Inhibited</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Erk-1/2</td>
</tr>
</tbody>
</table>

¹ Measures HGF-stimulated migration across gap in monolayer.
² Degrees inhibition of phosphorylation observed for protein listed in column.
³ Inhibition of number of colonies in soft agarose (colony: > 10 cells).
⁴ Compound induced apoptosis in presence and absence of HGF.
⁵ Differentiation describes flattened morphology and retained cell contact.

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Table 3 of the original document provides a summary of the effects of PHA-665752 on cell function and signaling in various cell lines. The table categorizes the effects on colony growth, cell proliferation, cell survival, cell migration, and cell morphology in response to PHA-665752 treatment. The effects are compared with the signaling molecules impacted, including Erk, Akt, FAK, PLC-γ, and STAT-3. The table outlines the specific cellular responses in each cell line, highlighting the selective inhibition of c-Met and its downstream effects on multiple signaling pathways.
ERKs 1 and 2, which is consistent with the mild mitogenic response to HGF and inhibition by PHA-665752 (Fig. 4).

### Inhibition of c-Met Phosphorylation in Tumor Xenografts in Vivo by PHA-665752 and Antitumor Efficacy in c-Met-Dependent Tumor Models

After demonstration of the inhibition of c-Met-dependent phosphorylation and cellular response to PHA-665752 in vitro, experiments were performed to evaluate its in vivo activity. S114 and GTL-16 models (s.c. tumor xenografts implanted in the hindflank of athymic mice) were selected for in vivo pharmacodynamic and efficacy studies because of the dependence on c-Met for cell growth in vitro and demonstration of constitutive phosphorylation of c-Met in tumors in vivo.

An initial study was designed to demonstrate pharmacodynamic inhibition of c-Met phosphorylation by PHA-665752 in tumors in vivo, and to determine the extent and duration of inhibition of c-Met phosphorylation after a single i.v. bolus dose of compound. As shown in Fig. 5A, after a single i.v. dose of PHA-665752 at 25 mg/kg to mice bearing S114 xenografts (approximately 300–400 mm³), c-Met phosphorylation (pY1230/34/35) in S114 tumors was potently inhibited through 12 h and returned to basal levels by 18 h. Similar patterns were observed in subsequent studies.

After demonstration of pharmacodynamic response, repeat-dose antitumor efficacy and pharmacodynamic studies were conducted in the S114 xenograft model over a range of doses. Mice bearing small, established S114 xenografts (~50 mm³) were administered PHA-665752 by daily i.v. bolus at 30, 15, or 7.5 mg/kg/day for 9 days. As shown in Fig. 5B, PHA-665752 demonstrated statistically significant dose-dependent tumor growth inhibition of 68%, 39%, and 20% of vehicle control at the 30, 15, and 7.5 mg/kg/day doses, respectively. The animals remained healthy and continued to gain weight during the 9-day treatment cycle. In addition, the extent of inhibition of c-Met phosphorylation in mouse B16-F1 melanoma cells (Table 1) indicates that inhibition of c-Met would be expected in mouse tissues. As shown in Fig. 5C, consistent with dose-dependent tumor growth inhibition, dose-dependent inhibition of c-Met phosphorylation in S114 tumors was observed at 4 h after dose on the final day of the study.

Studies designed to investigate effects of PHA-665752 on pharmacodynamic and tumor growth response in the nonengineered GTL-16 model were also conducted. Interestingly, repeat-dose i.v. administration of PHA-665752 at 25 mg/kg resulted in significant reduction in mass of large, established (~450 mm³) GTL-16 tumors, whereas the mass of vehicle-treated tumors continued to increase over the 9-day treatment schedule (Fig. 6A). A marked reduction in tumor volume was observed as early as 4 days after repeat-dose administration and was >40% smaller than the starting tumor mass by the end of the study. As shown in Fig. 6B, the antitumor effect of PHA-665752 was consistent with complete pharmacodynamic inhibition of c-Met phosphorylation at 4 h after dose as early as day 1 of the study. Similar to S114 studies, evaluation of PHA-665752 in subsequent experiments in the GTL-16 model demonstrated sustained inhibition of c-Met phosphorylation through 12 h after a single dose. In contrast to the S114 model, repeat-dose i.v. administration of 30, 15, and 7.5 mg/kg/day each demonstrated a similar potent antitumor effect with minimal evidence of dose-response in an independent GTL-16 study over 14 days (data not shown). These are the first studies demonstrat-
ing cytoreductive activity or tumor growth inhibition by a selective small molecule c-Met inhibitor.

**Immunohistochemical Evaluation of c-Met Phosphorylation, Signal Transduction, and Tumor Pathobiology in GTL-16 Xenografts After Treatment with PHA-665752.** After the observation of cytoreductive activity in the GTL-16 tumor model, studies were performed to evaluate the ability of PHA-665752 to: (a) inhibit c-Met activity and markers of mechanism and signal transduction at early time points; and (b) impact tumor pathobiology at later time points. In this evaluation, GTL-16 tumor-bearing mice were administered PHA-665752 for up to 8 days, and tumors from each treatment group (3 mice/group) were removed and fixed at 4-h after dose at days 1, 2, 4,

![Fig. 4. Effect of PHA-665752 on c-Met phosphorylation and signal transduction in GTL-16, NCI-H441, and BxPC-3 cells. Cells were treated with PHA-665752 (0.1 μM) for 3 h and with HGF (50 ng/ml) for 10 or 30 min. Lysates were made for each cell under each condition, and immunoblotting was performed for each protein or phosphoprotein of interest using specific antibodies as described in "Materials and Methods." Total Gab-1 was immunoprecipitated from protein lysates before immunoblotting. Representative blots from two independent experiments are shown in Fig. 4.](image1)

![Fig. 5. Inhibition of c-Met phosphorylation (A and C) and tumor growth (B) in the S114 tumor xenograft model. A, athymic mice bearing S114 tumors (300–400 mm³) were given a single i.v. dose (25 mg/kg) of PHA-665752 or vehicle. At several time points after administration, mice were euthanized, plasma and tumor samples were collected, and lysates were prepared as described in "Materials and Methods." The relative abundance of phospho-c-Met (pY1230/34/35) and total c-Met in the tumor lysates was determined by immunoblot analysis. Additional experiments in S114 tumors with PHA-665752 exhibited similar results. B, athymic mice bearing established (~50 mm³) S114 tumors were administered daily i.v. doses of PHA-665752 (7.5, 15, 30 mg/kg/day) or vehicle starting on day 7 through day 16. At least 10 mice were included in each group. Results shown indicate mean tumor volume; bars, ±SE. C, on day 16 of the efficacy study in 6B, at 4 h after final administration of PHA-665752, mice bearing S114 tumors (3/group) were euthanized, plasma and tumor samples were collected, and lysates were prepared for analysis of phospho-c-Met (pY1230/34/35) and total c-Met by immunoblot analysis at each dose.](image2)
and 8 of treatment. As early as 4 h after the first administration of PHA-665752, the inhibition of c-Met, ERK 1 and 2, Akt, and FAK phosphorylation was observed in fixed tumor sections by immunohistochemistry compared with vehicle-treated controls, and effect was sustained throughout the treatment duration (Fig. 7). The inhibition of the ERK 1/2 and Akt pathways is consistent with the reported role of these pathways in the regulation of tumor growth and apoptosis.

Because of these findings, and the inhibition of cell proliferation and induction of apoptosis observed in cultured GTL-16 cells, markers of tumor cell proliferation (Ki67) and apoptosis (activated caspase-3) were evaluated at 4 h after dose on days 1–7. However, only regional (exterior tumor rim versus interior) inhibition of Ki67 labeling and induction of activated caspase-3 was observed in GTL-16 tumors at the time points evaluated, making it difficult to determine the significance of these findings (data not shown). It is also possible that the narrow postdose time points selected in these studies were not appropriate to capture the optimal timing of these events. Evaluation of H&E sections indicated that PHA-665752-treated tumors over the dosing schedule exhibited increasingly marked necrotic regions in the interior of the tumor with viable tissue on the outer rim as compared with vehicle-treated tumors (Fig. 7). These observations indicate that extensive cell death occurred in the interior of the tumor throughout this study. However, the exact mechanism and/or timing of tumor cell death and cytoxreductive activity are not known.

**DISCUSSION**

The validity of therapeutic approaches to target RTKs that are dysregulated in human cancers are illustrated by the successes of Gleevec targeting bcr-abl in chronic myelogenous leukemia and c-Kit in gastrointestinal stromal tumors, Herceptin in HER-2 overexpressing breast cancers, and Iressa in select non-small cell lung cancers (1). The c-Met RTK has similar compelling evidence supporting its role in cancer and, hence, development of therapies that inhibit c-Met function. This supporting evidence includes: (a) the mutation, gene amplification, or overexpression of c-Met in a variety of cancers; (b) tumorigenic and metastatic properties of cell lines engineered to express c-Met and HGF; (c) development of tumors of multiple origins in c-Met and HGF transgenic animals; and (d) the inhibition of...
tumorogenic and metastatic properties of tumor cells using a variety of antagonists of c-Met function such as c-Met ribozymes, antisense, dominant-negative receptors, and NK4 (40). Here, we demonstrated that a selective small-molecule inhibitor targeting the catalytic activity of c-Met inhibits the oncogenic (proliferation, apoptosis, and anchorage-independent growth), prometastatic properties (motility and invasion), and signaling of c-Met in vitro, and pharmacodynamic inhibition of c-Met and antitumor efficacy, including tumor regression, in vivo.

Consistent with the reported pleiotropic effects of HGF and signaling through c-Met, PHA-665752 exhibited cell-type-dependent effects on multiple cellular functional endpoints. In the present studies, PHA-665752 exhibited inhibitory effects on cell growth and/or proliferation to varying degrees on GTL-16, NCI-H441, and BxPC-3 cells. However, differential effects of HGF or PHA-665752 on cell migration (only NCI-H441), cell morphology (only GTL-16 cells), and cell survival (only GTL-16 cells) were observed (summary in Table 3). Additional cell lines were identified in which PHA-665752 did not inhibit cell growth endpoints, but did affect an HGF-dependent motogenic response (e.g., AsPC-1 pancreatic and A549 non-small cell lung; data not shown). The regulation of different functions in different cancer cell types by c-Met is consistent with the cell-type and time-dependent coordinated regulation of cell migration, growth, and survival during organ morphogenesis in mammalian development (13, 15, 41–43). These observations indicate that the role of c-Met in progression of cancers may differ depending on the genetic context or lineage of a given tumor cell, which will be an important consideration in clinical development of c-Met-targeted therapies.

The roles of docking proteins and signaling mediators in regulation of diverse c-Met-dependent cell functions have been elucidated in studies using genetic, biological, and pharmacological means of selective modulation of these pathways. In the present studies, we evaluated the correlation of modulation of c-Met function to modulation of signal transduction molecules in each cell line with a distinct response. In each of the cell lines evaluated in these studies, PHA-665752 inhibited c-Met tyrosine phosphorylation at the activation loop (pY1230/34/35), multifunctional docking site (pY1349), and the juxtamembrane domain (pY1003) at 0.1 μM. Consistent with the inhibition of c-Met phosphorylation at the multifunctional docking site, total tyrosine phosphorylation of Gab-1, the c-Met adaptor protein that binds to this site, was completely inhibited in all of the cell lines evaluated. The consistent modulation of Gab-1 tyrosine phosphorylation by PHA-665752 in these cell lines, and others not reported here is consistent with the reported requirement for Gab-1 in the mediation of critical c-Met-dependent cellular functions and the relative specificity for c-Met compared with other RTKs for sustained activation of Gab-1 (43).

Because downstream signal transducers evaluated in these studies are also activated by other RTKs, as expected, signaling mediators further downstream of c-Met were regulated in a cell-specific manner. One common effect observed was the inhibition of constitutive or HGF-stimulated ERK phosphorylation in each cell line evaluated. The activation of ERKs through the Grb2-SOS-Ras cascade has been linked with uncontrolled cell proliferation, consistent with the regulation of cell growth in each of the cell lines evaluated. Studies with the ERK pathway inhibitor PD98059 have also indicated that sustained activation of this pathway is required but not sufficient for HGF-dependent motogenesis and morphogenesis (44–46). Inhibition of constitutive Akt phosphorylation was observed in GTL-16 and H441 cells treated with PHA-665752, indicating the modulation of the PI3K pathway. Studies with inhibitors such as wortmannin and LY294002 have indicated that the PI3K pathway is both required and sufficient for HGF-dependent branching morphogenesis under certain conditions, and is required for motility and cell survival (44, 47–49). These findings support the role of the inhibition of the PI3K pathway by PHA-665752 in regulation of morphogenetic differentiation and survival of GTL-16 cells, motility of H441 cells, and lack of these responses in BxPC-3 cells. However, Akt inhibition is observed in both GTL-16 cells and H441 cells with potentially different consequences on cell response. The cell context under which c-Met differentially regulates survival, morphogenesis, and motogenesis through PI3K is not completely understood; however, durability of signaling pathway activation and signaling cross-talk with the ERK pathway have been described as mediators of differential response (44, 47, 49, 50).

FAK and c-Src are described as being required for loss of intracellular junctions and gain of cell-matrix adhesion during HGF-dependent motility and scatter responses (38, 39). In the present studies, phosphorylation of the c-Src-dependent regulatory site of FAK (pY-861) was the only phospho-protein markedly induced by HGF in NCI-H441 cells, whereas other phospho-proteins that were modulated were constitutively activated. Although PHA-665752 inhibited FAK phosphorylation in both GTL-16 and NCI-H441 cells, a motility response to HGF was only observed in NCI-H441 cells. This suggests the possibility that the induction of FAK activity is a rate-limiting step in the motogenic process in these cells. In addition, inhibition of constitutive FAK activity by PHA-665752 in GTL-16 cells suggests a role in morphological change and gain of cell-cell contact observed. The inhibition of STAT-3 and PLC-γ phosphorylation only in GTL-16 cells is also consistent with the requirement for these proteins in branching morphogenesis and morphological change (41, 51). Collectively, the differential regulation of signaling mediators by PHA-665752 in a cell-specific manner is consistent with the differential regulation of cellular functions by c-Met. The understanding of the relationship of regulation of signal transduction to cell function has the potential to facilitate the interpretation of the functional role of c-Met in monitoring the response of a given tumor type to c-Met-targeted therapies.

After demonstrating that inhibition of c-Met phosphorylation by PHA-665752 resulted in tumor growth inhibition in the c-Met/HGF engineered S114 model, antitumor and antinmetastatic activity was demonstrated in other models. The present studies demonstrating in vivo pharmacodynamics and antitumor efficacy by PHA-665752 begin to demonstrate a correlation between the extent of inhibition of c-Met phosphorylation and inhibition of tumor growth. In addition, the demonstration of inhibition of c-Met in mouse cells and the fact that the animal remained healthy during treatment suggests that an adequate therapeutic index may be possible with a selective inhibitor. Together, these results support the utility of measuring c-Met phosphorylation as a pharmacodynamic marker in the monitoring of antitumor efficacy and safety.

Interestingly, inhibition of c-Met activity by PHA-665752 resulted in cytoreductive activity in the GTL-16 tumor model. GTL-16 contains an amplified c-Met gene locus resulting in expression of high levels of constitutively active c-Met. These data suggest that c-Met may potentially play a role as a survival factor in selected tumors. These tumor types may include those where the receptor is altered by events that result in high constitutive activity, such as gene amplification or mutations, consistent with the precedent set by Gleevec and Herceptin. PHA-665752 inhibited proliferation and induced apoptosis in cultured GTL-16 cells, supporting its role as a survival factor in these cells. In addition, PHA-665752 inhibited ERK, Akt, and FAK phosphorylation in both cultured GTL-16 cells and tumors, suggesting similar alterations in signaling pathways that regulate tumor cell growth and survival both in vitro and in vivo. The evaluation of tumor H&E sections indicated treated tumors exhibited comparatively
marked necrotic regions on the interior of the tumor, indicating that extensive cell death occurred in this region of the tumor during PHA-665752 treatment. Collectively, these data support the role of c-Met as a survival factor in selected tumors. Studies to further evaluate c-Met inhibitors in additional models of tumor growth and metastasis, and the correlation of the extent and duration of inhibition of c-Met phosphorylation to antitumor efficacy are warranted and ongoing.

In summary, these studies illustrate the effects of a selective c-Met inhibitor, PHA-665752, as an inhibitor of a variety of c-Met-dependent functions and signaling events. These studies are the first demonstration of inhibition of c-Met phosphorylation and antitumor efficacy, including cytoressive activity, by a selective, small-molecule c-Met inhibitor. The diversity of response to PHA-665752 in a variety of cell lines indicates the variety of roles that c-Met plays in different cells and, putatively, tumor types. These results suggest that the role of c-Met in tumorigenesis and progression of cancers may differ depending on the genetic context of a given tumor cell. In addition, the understanding of the relationship of regulation of signal transduction to cell function has the potential to facilitate the interpretation of the functional role of c-Met in monitoring the response of a given tumor type to c-Met targeted therapies. The diverse role of c-Met in different tumor types, as a regulator of tumor growth, survival, or metastasis, will be a critical issue in the clinical development of c-Met inhibitors, monitoring of patient response, and design of clinical studies.

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A Selective Small Molecule Inhibitor of c-Met Kinase Inhibits c-Met-Dependent Phenotypes \textit{in Vitro} and Exhibits Cytoreductive Antitumor Activity \textit{in Vivo}

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