A Selective Small Molecule Inhibitor of c-Met, PHA665752, Inhibits Tumorigenicity and Angiogenesis in Mouse Lung Cancer Xenografts


Abstract

The c-Met receptor tyrosine kinase is emerging as a novel target in many solid tumors, including lung cancer. PHA-665752 was identified as a small molecule, ATP competitive inhibitor of the catalytic activity of the c-Met kinase. Here, we show that treatment with PHA665752 reduced NCI-H69 (small cell lung cancer) and NCI-H441 (non–small cell lung cancer) tumorigenicity in mouse xenografts by 99% and 75%, respectively. Reduction in tumor size was also observed by magnetic resonance imaging of tumors in mice. PHA665752 inhibited c-Met phosphorylation at the autophosphorylation and c-Cbl binding sites in mouse xenografts derived from non–small cell lung cancer cell lines (NCI-H441 and A549) and small cell lung cancer cell line (NCI-H69). PHA665752 also inhibited angiogenesis by >85% in all the abovementioned cell lines and caused an angiogenic switch which resulted in a decreased production of vascular endothelial growth factor and an increase in the production of the angiogenesis inhibitor thrombospondin-1. These studies show the feasibility of selectively targeting c-Met with ATP competitive small molecule inhibitors and suggest that PHA665752 may provide a novel therapeutic approach to lung cancer. [Cancer Res 2007;67(8):3529–34]

Introduction

There will be >213,000 cases of lung cancer and >160,000 deaths from lung cancer in 2007 (1). Unfortunately, even with the best of therapies, the overall survival for non–small cell lung cancer (NSCLC) is 16%, whereas for small cell lung cancer (SCLC), overall survival is 6% (1). In order to enhance the survival for patients with lung cancer, basic molecular mechanisms have to be discerned with the potential for novel therapeutics. Receptor tyrosine kinases (RTK) seem to be an important therapeutic target in malignancies (2).

c-Met is a RTK that is expressed during normal development and can be altered in a number of malignancies. The ligand for c-Met is the hepatocyte growth factor (HGF), and activation of c-Met with HGF can lead to cell growth and viability, cell motility, angiogenesis, as well as transformation of cells (3). c-Met can not only be activated via its ligand, but also through amplification or mutation (3). We have previously shown that c-Met is overexpressed in 67% adenocarcinomas and 35% of SCLC (4, 5). Approximately 10% to 15% of adenocarcinomas of the lung and SCLC have a mutation in the semaphorin or juxtaplumembrane domains of lung cancer, and not in the tyrosine kinase domain (4, 5). Activation of c-Met in lung cancer leads to enhanced cell proliferation, increased cell motility, increased production of reactive oxygen species (6), and activation of several signal transduction pathways such as the focal adhesion and phosphatidylinositol-3'-kinase/Akt pathway (4, 5). Because c-Met has a large effect on biological and biochemical signaling, it is important to particularly target this pathway.

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 (7). 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 in clusters as metastatic tumors in nude mice (8, 9). In addition, transgenic mice overexpressing c-Met, HGF, or mutant c-Met display a tumorigenic and metastatic phenotype (10, 11).

The clinical therapeutic targeting of c-Met is just beginning to come to fruition. There are several strategies to inhibit the HGF/c-Met axis. Small interference RNA (siRNA) against c-Met has been used in lung cancer cell lines with reduced cell growth and enhanced apoptosis (4). Antibodies such as 5D5 against c-Met have also been shown to be effective in cell line models (12). Most recently, small molecule inhibitors such as SU11274 and PHA665752 have been used against lung cancer cell lines in tissue cultures with considerable success (4, 5). Treatment of lung cancer cell lines with SU11274 and PHA665752 leads to decreased cell growth, decreased cell motility, decreased production of reactive oxygen species, as well as eventual apoptosis (4, 5). Interestingly, Smolen et al. (13) have recently found that cancer cells with high levels of stable chromosomal amplification of c-Met are extraordinarily susceptible to the selective inhibitor PHA-665762. At this time, animal models to inhibit c-Met have not been tested against lung cancer and would be an effective screening approach in the preclinical development of targeted drugs.

In this study, we have evaluated the effects of the specific c-Met inhibitor, PHA665752, against the mouse models of H69 SCLC and H441 NSCLC. We find specific reduction of tumor growth with PHA665752 in the lung cancer models. This was also reflected in radiological imaging with magnetic resonance imaging (MRI). We also find that treatment with PHA665752 resulted in a decrease in the number of blood vessels (as assessed with CD31 staining) and a switch in the angiogenic phenotype which resulted in a decrease in the production of vascular endothelial growth factor (VEGF) and...
an increase in the naturally occurring inhibitor of angiogenesis thrombospondin-1 (TSP-1). With these types of studies to show the importance of therapeutically targeting c-Met in lung cancer, we would predict that this would be important to bring to clinical fruition.

Materials and Methods

Chemicals, Cells, and Antibodies

PHA-665752 (3Z)-5-[(2,6-dichlorobenzyl)sulfonyl]-3-[(3,5-dimethyl-4-1H-pyrrol-2-yl)methylene]-1,3-dihydro-2H-indol-2-one (obtained from Pfizer Inc., San Diego, CA) was suspended in DMSO and kept in small aliquots at –20°C and used at the concentrations described. Phosphospecific antibodies to c-Met pY1230/1234/1235 (autophosphorylation site) and pY1003 (c-Cbl binding site) were obtained from Biosource International (Camarillo, CA). CD31 antibody (sc 1506), VEGF antibody (sc 152), and TSP-1 (sc 12312) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NCI-H441, A549, and NCI-H69 cells were obtained from American Type Culture Collection (Rockville, MD) and were cultured according to the instructions.1

Figure 1. PHA665752 injected into NCI-H69–derived mouse xenografts reduces tumor size. NCI-H69 cells (5 × 105) were injected s.c. into the leg of nude mice. There were 10 mice each in treated and control groups. After 8 d, when tumor nodules were clearly visible, sites were injected once daily with PHA665752 (16.5 μg in 100 μL of 2% DMSO) or 100 μL of 2% DMSO. Control animals and animals treated with PHA665752 were euthanized after 21 d and tumors were excised, measured, and processed for immunostaining. A, mice in control groups with large tumors (left), and animals treated with PHA665752 with no visible tumors (right). Five out of 10 animals had no detectable tumors in the PHA665752-treated group and tumors in the remaining five animals were strikingly smaller. B, MRI of mice treated with PHA665752 for 3 wks. Control animals (left) and treated tumors (right). Tumors were imaged and spin echo images were acquired with a 4.7 T animal scanner. MRI show only a small residual tumor mass in PHA665752-treated animals. C, PHA665752 injected into NCI-H69–derived mouse xenografts results in smaller or undetectable tumors. H&E staining of untreated NCI-H69 tumor xenografts (left) showing presence of tumor tissue. H&E staining of NCI-H69 tumor treated for 3 wks with PHA665752 (right) showing connective tissue elements and absence of tumor. D, 10 mice with NCI-H69 xenografts were injected with PHA665752 and 10 mice were in a control group as described above. Treatment with PHA66572 reduced tumor size by 99% in comparison with control-treated animals. E, four mice with NCI-H441 xenografts were injected with PHA665752 (16.5 μg in 100 μL of 2% DMSO) and four control mice were injected with 100 μL of 2% DMSO for 2 wks. Treatment with PHA665752 reduced tumor size by 75%.

1 http://www.ATCC.org
Animals

Five-week-old male Ncr-nu (Nude) mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the pathogen-free animal facility at the University of Chicago. Animal treatments were done according to institution-approved protocols.

Treatment of Nude Mice for Studying the Effect of c-Met Tyrosine Kinase Inhibitors on Tumorigenicity and Immunohistochemistry of Tumors

NCI-H441, A549, and NCI-H69 cells were cultured and harvested with trypsin/EDTA. The viability of these cells was determined by trypan blue and only cell populations with 90% or greater viability were used for this investigation. The tumorigenicity of these lung cancer cells was determined by intradermally injecting $5 \times 10^6$ viable cells in balanced salt solution into the flank or leg region of nude mice to produce s.c. tumors. Once daily intratumoral injections of PHA665752, were given 8 days after the lung cancer cells were injected when tumors were visible. One group of animals was given PHA665752 (16.5 μg in 100 μL of 2% DMSO) and group 2 was injected with diluent (2% DMSO) alone. The mice were euthanized at the indicated times and tumors were measured with calipers, fixed in 4% formalin, embedded in paraffin, and stained with H&E.

Immunohistochemical staining of tumors was done using monoclonal antibodies against CD31, VEGF, TSP-1, and phosphospecific antibodies to c-Met pY1230/1234/1235 and pY1003. The immunostaining procedures used have been previously described by Ma et al. (4). Appropriate negative controls for the immunostaining were prepared by omitting the primary antibody step and substituting it with nonimmune rabbit serum. To estimate the number of blood vessels in tumors before and after treatment,
10 microscopic fields were counted at 20× magnification and the number of blood vessels were evaluated. All of the slides were reviewed and scored by two investigators independently.

MRI Protocol
Animal preparation. Mice were anesthetized with 1.5% to 2% isoflurane. A SA Instruments (Brooklyn, NY) physiologic monitoring system was implemented to acquire electrocardiogram and respiratory signals for motion gating. Body temperature was maintained at 38°C with a resulting 400 to 450 cardiac rate.

MRI. All MRI experiments were conducted on a Bruker 4.7 T animal scanner with 20 G/cm gradients. Control and treated H69 tumors were imaged and spin echo images were acquired with a 4.7 T animal scanner with the following variables: TR/TE = 1,500/10 to 20 ms, FOV = 3 cm, array size = 256 × 256, slice thickness = 1.0 mm, NEX = 1.

Results

Effect of PHA665752 on lung cancer tumor size in nude mice. In order to determine whether PHA665752 can affect the growth of preexisting tumors, 5 × 10⁶ NCI-H69 cells which are derived from SCLC were injected s.c. into the flank of nude mice. Beginning 8 days later, when small tumor nodules were apparent, the sites were injected once daily with PHA665752 (16.5 μg in 100 μL of 2% DMSO) or an equal volume of 2% DMSO alone. The tumors were injected intratumorly because the drug is sparingly soluble in water and we were interested in delivering an effective dose directly into the tumor, and in comparing the results of intratumoral delivery with the results of Christensen et al. (7), who gave the drug i.v. to gastric carcinomas. The dose of PHA665752 used in this study was 0.825 mg/kg/d, a dose much lower than used by the study from Christensen et al. (7), in which a dose of 15 to 30 mg/kg/d was used and was found to be a more effective inhibitor of tumor growth. After 3 weeks, animals were euthanized and the tumor size was determined. There was a very significant difference in tumor size between diluent and PHA665752-treated mice (Fig. 1A and B), and tumors were clinically undetectable in 5 out of 10 mice, as seen by immunohistochemistry, which shows the complete absence of tumors at the treated site (Fig. 1C). The tissue surrounding the injected tumor nodules appeared normal in all animals. PHA665752 treatment reduced tumor volume (P < 0.0001) by 99% as determined by measurement of the tumors with calipers (Fig. 1D) and by an animal MRI scanner (Fig. 1B).

The effect of treatment with PHA665752 was also studied in tumor xenografts from NCI-H441 and A549, which were derived from NSCLC. NCI-H441 cells were treated as described above and it
was found that tumor growth was inhibited by 75% (Fig. 1E). Treatment with PHA665752 also inhibited the growth of A549 cells by 59% (data not shown).

**Immunohistochemistry and angiogenesis of tumors.** PHA665752 treatment reduced the expression of phospho c-Met pY1230/1234/1235 (autophosphorylation site) and pY1003 (c-Cbl binding site) in the small residual tumor nodules (Fig. 2) in both NCI-H69 (Fig. 2A) cells and A549 cells (Fig. 2B). These results indicate that reduction in c-Met phosphorylation has a very significant effect on tumor proliferation.

Treatment with PHA665752 also resulted in >85% reduction (Fig. 3A–C) in the number of blood vessels seen by CD31 staining in NCI-H441, A549, and NCI-H69 indicating that inhibition of vessel formation may be one of the mechanisms by which PHA665752 inhibits tumor growth. We further immunostained H69 tumors for the expression of VEGF and TSP-1. We found a statistically significant up-regulation of TSP-1 (Fig. 4A) and a concomitant down-regulation of VEGF (Fig. 4B). These results indicate that a switch from an angiogenic to an angiinhibitory phenotype is one of the potential mechanisms by which treatment with PHA665752 resulted in a change in the angiogenic status of NCI-H69 tumor xenografts.

**Discussion**

c-Met is an important therapeutic target against lung cancer (4, 5). With the dismal prognosis of lung cancer, it is crucial to discover novel targets to ultimately bring to clinical fruition. In this study, we show that in both SCLC and NSCLC xenograft nude mouse models that there was significant tumor growth reduction after treatment with c-Met–specific small molecule inhibitor PHA665752. We have previously published in cell line models that PHA665752 is a specific inhibitor of c-Met with an IC₅₀ of 0.06 μmol/L. PHA665752 also synergized with mTOR inhibitor rapamycin to cause apoptosis (14). We also show that there is decreased activation of c-Met with this specific inhibitor via immunohistochemistry. The angiogenic properties of the c-Met/HGF axis are well known (3) and can result in a critical angiogenic switch by Shc for VEGF production (15). We provide the first evidence of antiangiogenic effects of inhibiting c-Met in the mouse model with PHA665752 and show for the first time that treatment with PHA665752 results in a change in the angiogenic phenotype resulting in an increase in the production of angiogenesis inhibitor TSP-1 with a concomitant decrease in VEGF production.

Small molecule inhibitors, specifically against c-Met, represent an attractive novel targeted therapeutic approach. We have reported, for the first time, the effectiveness of a novel small molecule–specific inhibitor of c-Met, SU11274 (Pfizer; previously Sugen), in cells transformed by the oncogenic Tpr-Met as a model, as well as in SCLC and NSCLC (4, 16). Inhibition of the Met kinase activity by the drug SU11274 led to time- and dose-dependent reduced cell growth, and induced G₁ cell cycle arrest and apoptosis. Met kinase autophosphorylation was reduced on sites that have been previously shown to be important for the activation of pathways involved in cell growth and survival, especially the phosphatidylinositol-3'-kinase and the Ras pathway (16). The characterization of SU11274 as an effective inhibitor of Met tyrosine kinase activity illustrates the therapeutic potential of targeting Met in cancers associated with activated forms of this kinase. Similar results were seen with PHA665752 in a study by Ma et al. (4). The use of siRNA is another novel approach to study the effects of inhibition on RTKs (4). In lung cancer CL-1 cells, siRNA has been used to inhibit the membrane-anchored glycoprotein RECK and has shown that histone-deacetylase inhibitors suppress tumor invasion with their inhibitory effect on MMP-2 activation mediated via RECK (17).

In lung cancer, we have identified that there are specific mutations/alterations of the c-Met gene in the juxtamembrane domain, semaphorin domain, and an alternative spliced product at exon 10 (4, 5). The juxtamembrane domain serves as a negative
regulator of the tyrosine kinase domain, and once mutated, there is activation of the tyrosine kinase domain of c-Met. Interestingly, H69 SCLC used in the above study harbors an R988C mutation in the juxtamembrane domain (5) and thus may be more responsive to the PHA665752. Juxtamembrane domains of RTKs are thought to be key regulators of catalytic functions (18).

Besides missense mutations, c-Met–mediated tumorigenesis can be a result of gene amplification, as seen in human gastric carcinoma via the break-fusion-bridge mechanism (19). In gastric cancer cell lines with c-Met amplification, it has also been shown that treatment with PHA-665752 triggers massive apoptosis (13). As we discern specific mutations of c-Met in the context of various tumors, it would be useful to determine the effect of c-Met inhibition with the wild-type and mutated forms of c-Met in a mouse model. Ultimately, it is also suspected that some tumor cells may escape the vigilance of c-Met inhibition, which may lead to resistance. Thus, for lung cancer, which is responsive to cytotoxic chemotherapies such as cisplatin, paclitaxel, docetaxel, pemetrexed, and erlotinib it would be useful to combine the novel c-Met–targeted therapy with these agents.

In this study, we show that c-Met can be targeted via the small molecule inhibitor PHA665752 in lung cancer through xenograft mouse models. It would now be useful to bring to clinical fruition compounds such as PHA665752 against lung cancer.

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