A selective small molecule inhibitor of c-Met, PHA-665752, reverses lung premalignancy induced by mutant *K-ras*

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Abstract

The c-Met receptor tyrosine kinase has been implicated in cellular transformation induced by mutant *Ras*, a commonly activated proto-oncogene in non-small cell lung cancer (NSCLC). However, the role of c-Met has not been defined in *K-ras*-mutant NSCLC, a disease for which no effective targeted therapeutic options currently exist. To acquire a greater understanding of its role, we used genetic and pharmacologic approaches to inhibit c-Met in mice and cultured cells. In $Kras^{LA1}$ mice, which develop premalignant lung lesions that progress to multifocal lung adenocarcinomas owing to somatic mutations in *K-ras*, c-Met was expressed in multiple cell types within premalignant lung lesions, and high concentrations of HGF were detected in bronchoalveolar lavage samples. Short-term treatment with PHA-665752, a c-Met inhibitor, decreased the numbers of premalignant lung lesions and induced apoptosis in tumor cells and vascular endothelial cells within lesions. In cell culture, PHA-665752 induced apoptosis of a lung adenocarcinoma cell line derived from $Kras^{LA1}$ mice (LKR-13) and a murine lung endothelial cell line (MEC). c-Met depletion by siRNA transfection induced apoptosis of MECs but not LKR-13 cells. Collectively, these findings suggest that apoptosis was an on-target effect of PHA-665752 in MECs but not in LKR-13 cells. We conclude that PHA-665752 inhibited lung tumorigenesis in $Kras^{LA1}$ mice and may provide a novel therapeutic approach to the prevention of *K-ras*-mutant NSCLC. [Mol Cancer Ther 2008;7(4):952–60]

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Introduction

Non-small cell lung cancer (NSCLC) is the most frequent cause of cancer-related death in the United States, a result, in part, of a dearth of effective treatments for patients with advanced NSCLC and for individuals at high risk for the development of this disease (1). Recent advancements in this area include the identification of activating somatic mutations in the *epidermal growth factor receptor* (*EGFR*) and evidence that patients with *EGFR*-mutant NSCLC frequently experience rapid tumor shrinkage in response to treatment with EGFR TKIs (2). However, *EGFR* mutations occur in only 10% of NSCLC patients in this country, and effective therapeutics are needed for patients with NSCLC harboring other somatic mutations, such as mutant *K-ras*, which accounts for 20-30% of NSCLC (3). No effective targeted therapeutic approaches are currently available for patients with *K-ras*-mutant NSCLC.

Addressing this deficiency for patients with *K-ras*-mutant NSCLC will require improvements in our understanding of the fundamental mechanisms by which *K-ras* mutations promote lung tumorigenesis. As one possibility, cells transformed by mutant *Ras* have been shown to be dependent upon the c-Met receptor tyrosine kinase for invasion and metastasis (4). c-Met contributes to a variety of cellular responses, including survival, proliferation, migration, invasion, and branching morphogenesis (5). Mechanistically, the binding of c-Met to its ligand hepatocyte growth factor (HGF) induces autophosphorylation of the cytoplasmic domain of c-met at residues that lead to kinase activation (Tyr-1234 and -1235) and the recruitment of adaptor proteins (Tyr-1349 and -1356) that activate downstream signaling molecules, such as Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase, phospholipase C-γ, the Shp2 protein tyrosine phosphatase, Crk/CrkL, and phosphoinositol 3-kinase, which is the primary mediator of cell survival induced by c-Met (5).

There is a growing body of evidence that c-Met is activated in NSCLC. c-*Met* is somatically mutated in intronic regions leading to a loss of Cbl E3-ligase binding, which enhances c-met protein stability in NSCLC cells (6). Other somatic mutations have been identified within the semaphorin and juxtamembrane domains of c-*Met* that constitutively activate c-met receptor tyrosine kinase activity and enhance the motility of NSCLC cells (7). In addition, c-Met is activated in NSCLC cells through

cell-autonomous mechanisms by tumor-infiltrating neutrophils and lung fibroblasts that express HGF and activate c-Met in a paracrine fashion (8–11). The net effect of the activation of c-Met through these mechanisms is that NSCLC cell proliferation and survival are maintained. Not surprisingly, therefore, the intratumoral HGF expression is a predictor of poor outcome in NSCLC patients (8).

In this study, we investigated the role of c-Met in lung neoplasia induced by mutant *K-ras*, an important question given the known association of Ras mutations with high c-Met expression, the frequency of *K-ras* mutations in NSCLC, and the current lack of effective therapeutic and preventive strategies for this disease. We tested the activity of PHA-665752, a selective small molecule inhibitor of c-Met (12), in $Kras^{LA1}$ mice, which develop lung adenocarcinomas as a result of a somatic mutation (G12D) of *K-ras* (13). Prior to the appearance of adenocarcinomas, the lungs have multiple premalignant lesions that are designated histologically as adenomatous alveolar hyperplasia (AAH) and adenomas that are heavily infiltrated with macrophages, neutrophils, and vascular endothelial cells (14, 15). We chose to study these mice at an early stage of tumorigenesis because inflammatory cells and stromal fibroblasts that infiltrate NSCLC are reported to secrete HGF (8–11). We found that PHA-665752 inhibited lung tumorigenesis in this model and concluded from these findings that additional preclinical studies are warranted to explore the efficacy of this and other selective c-Met inhibitors against *K-ras*-mutant NSCLC.

Materials and Methods

Reagents

LKR-13 cells and MECs were derived and maintained as previously described (15). We purchased rabbit polyclonal antibodies against human Factor VIII (DakoCytomation, Carpinteria, CA); SPC (Research Diagnostic, Inc, Concord MA); phosphorylated AKT (Ser 473), phosphorylated p42/44 MAPK (Thr 202/Tyr 204), phosphorylated c-met (Tyr1234/1235), total p42/44 MAPK, AKT, and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA); cleaved PARP and total c-met (Santa Cruz Biotechnology, Santa Cruz, CA); rat monoclonal antibodies against F4/80 and p40 (Serotec, Oxford, UK); and goat polyclonal antibodies against HGF and c-met (R&D Systems). For fluorescence staining, we used rabbit antibodies against cleaved caspase-3 (FITC; Cell Signaling Technology) and CD31 (PE; Research Diagnostic, Inc, Concord, MA) and secondary anti-rabbit and anti-rat antibodies (TRITC; Immunology Consultants Laboratory, Inc., Newburg, OR). Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole II (Vysis, Inc., Downers Grove, IL).

Animal Experiments

Animal experiments were done in compliance with the guidelines of The University of Texas M. D. Anderson Cancer Center. The $Kras^{LA1}$ mice were provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). Sixteen-week-old K-ras^{LA1} mice were given intravenous tail vein injections of the c-met inhibitor PHA-665752 (Pfizer) at a dosage of either 25 mg/kg/day (high-dose group, $n = 5$) or 12.5 mg/kg/day (low-dose group, $n = 5$) for 6 days in a volume of 150 μl; another 5 control mice were given vehicle (L-lactate [pH 4.8] and 10% polyethylene glycol). Treatment was limited to 6 days to avoid the venous toxicity associated with longer administration (12). At autopsy, the animals' lungs were perfused with PBS and removed from the body. One lung was kept at −80°C for protein extraction and the other was fixed in 4% paraformaldehyde for 30 minutes followed by 10% formalin overnight before being embedded in paraffin as previously described (15). To obtain the bronchoalveolar lavage specimens, a separate group of 16 week-old $Kras^{LA1}$ mice ($n = 9$) and wild-type littermates ($n = 6$) were killed by cervical dislocation, and then three 1-ml aliquots of PBS were injected directly into the trachea. The liquid was recovered by gentle aspiration and centrifuged. The supernatants were recovered and frozen at −80°C.

ELISA

To measure concentrations of HGF in bronchoalveolar lavage supernatants from $Kras^{LA1}$ mice ($n = 9$) and 129/sv wild-type littermates ($n = 6$), ELISA was performed according to the supplier's instructions (Institute of Immunology, Tokyo, Japan). Results were expressed as the mean concentration (pg/ml) ± standard error of the mean (SEM).

Murine Tissue Microarrays and Immunohistochemical Analysis

Microarrays were constructed of cores from the formalin-fixed, paraffin-embedded blocks of all lesions identified by histologic analysis from the mice treated with PHA-665752 (high or low dose) or vehicle. A single core of tissue 1 mm in diameter was obtained from each murine lesion. Four-micrometer-thick sections were deparaffinized, rehydrated, washed with PBS, and subjected to antigen retrieval and staining with primary and secondary antibodies, as previously described (14, 15). Negative controls for immunohistochemical staining consisted of the removal of primary antibody. Staining was quantified by two independent investigators (M. W. and I. I. W.), who were blinded to the treatment groups. Lesions were scored according to the frequencies of positive cells within lesions (cleaved caspase-3, F4/80, p40, Factor vIII) or a combination of staining intensity and extension (phosphorylated Ser473-AKT), as previously described (14, 15). Tissues were visualized at X20 magnification for the scoring with all antibodies, with the exception of cleaved caspase-3, which was visualized at ×40 magnification.

Immunofluorescence Studies

For the immunofluorescence studies, 4-μm-thick sections were deparaffinized, rehydrated, and washed with TBS-T. Antigens were retrieved by exposure to antigen retrieval buffer (DakoCytomation) for 30 minutes in a steamer. Samples were blocked for endogenous activity in 3% H^2O^2/TBS, avidin/biotin solution (Zymed, South San Francisco, CA) and DAKO serum-free protein block

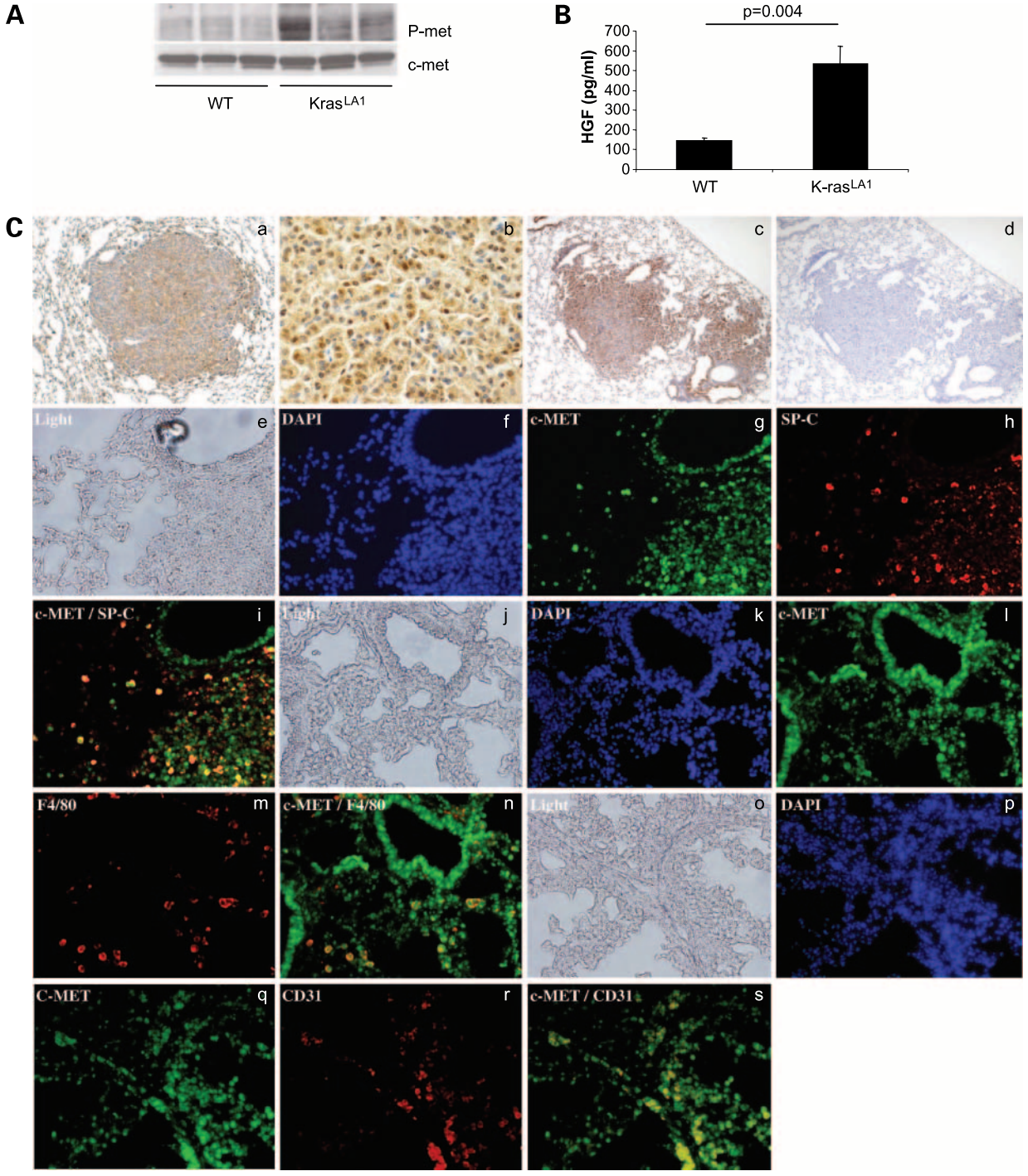

Figure 1. Analysis of c-Met phosphorylation and HGF concentration in lungs of Kras^{LA1} mice. **A,** Tyr-1234/1235-phosphorylation of c-Met in whole-lung lysates was higher in Kras^{LA1} mice than in wild-type (WT) littermates. Western blot analysis of Tyr1234-phosphorylated c-met (P-met) and total c-met performed on whole-lung lysates from Kras^{LA1} (n = 3) and wild-type (n = 3) littermates. **B,** comparison of HGF concentrations in bronchoalveolar lavage samples reveals higher levels in Kras^{LA1} mice (n = 9) than in wild-type (WT) littermates (n = 6). HGF concentrations were measured by ELISA (mean value ± S.E.M). **C,** c-Met was expressed in multiple cell types within premalignant lung lesions of Kras^{LA1} mice. Representative immunohistochemical stains for c-met in two lung adenomas (panels a-d). One adenoma illustrated at 4× (panel a) and 40× (panel b) magnifications to show the predominantly cytoplasmic staining, and the other adenoma stained in the presence (panel c) or absence (panel d) of primary antibody to demonstrate the specificity of staining (both illustrated at ×4 magnification). Dual-immunofluoresence staining was performed using antibodies against total c-met (panels g, l, and q) and markers for specific cell types, including surfactant protein-C (SPC) for tumor cells (panel h), F4/80 for macrophages (panel m), and CD31 for vascular endothelial cells (panel r). c-Met stains were merged with corresponding stains for SPC (panel i), F4/80 (panel n), and CD31 (panel s). Light microscopic fields (panels e, j, and o) and DAPI stains (panels f, k, and p) are illustrated. Panels e-s are ×10 magnification.

(DakoCytomation) before incubation with the primary antibodies overnight at 4°C. The slides were then washed with TBS-T and incubated with appropriate biotinylated secondary antibodies for 30 minutes. The fluorescence was developed using TSA kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Western Blot Analysis

LKR-13 cells and MECs were subjected to 24 hours of treatment with PHA-665752 at the indicated doses. Lysates of cell lines or mouse tissue containing 30 to 40 μg of protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to western blotting as previously described (14, 15).

Proliferation Assays

To measure sensitivity to PHA-665752 *in vitro*, LKR-13 cells or MECs were plated (1,000 cells/well) in quadruplicate in 96-well tissue culture plates and incubated for 24 hours before being exposed to different concentrations of PHA-665752 or vehicle (DMSO). Proliferation after 3 days of incubation was quantified by 3-(4,5 dimethylthiazol-2-thiazyl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) assays.

Apoptosis Assays

LKR-13 cells or MECs were treated for 24 h with PHA-665752 and then subjected to western blotting for cleaved caspase-3 or stained with bisbenzimide (Hoechst 33342), as previously described (14, 15). Nuclei were analyzed for chromatin condensation using a fluorescence microscope with a UV filter at a magnification of ×40.

c-Met siRNA Transfection

LKR and MEC cells were transfected with scrambled RNA or specific siRNA for c-Met (Dharmacon) using pooled siRNA oligomers (4 oligomers per pool at a final concentration of 80 pM) using the transfection reagents provided by the manufacturer (Invitrogen). After 72 h of transfection, the cell densities and the expression of c-Met and cleavage of caspase-3 and PARP were examined.

Statistical Analysis

Immunostaining scores, numbers of lung lesions, and results from proliferation and migration assays were considered continuous variables. The patients' clinical variables and tumor histologic features were summarized using standard descriptive statistics and frequency tabulation. Associations between categorical variables were assessed using chi-squared test and Fisher's exact test. The Kruskal-Wallis test was used to assess group differences for three-group comparisons and the Mann Whitney nonparametric test for two-group comparisons. P values of ≤ 0.05 were considered significant. Data were processed with StatView and Survival Tools 5.0 (Abacus Concepts, Berkeley, CA).

Results

c-Met Activation in the Lungs of $Kras^{LA1}$ Mice

We hypothesized that c-Met is required for the malignant progression of tumors in $Kras^{LA1}$ mice. Prior to testing this, we first examined evidence of c-Met activation in lung tissues by performing western blot analysis of c-Met phosphorylation on Tyr-1234/1235, an autophosphorylation site (5), in whole-lung lysates from $Kras^{LA1}$ mice ($n = 3$) and wild-type littermates ($n = 3$; Fig. 1A). Although total c-Met levels were not different, densitometric scanning revealed, on average, 3-fold higher c-met phosphorylation in $Kras^{LA1}$ mice than in controls. To investigate whether the higher phosphorylation might be a consequence of increased concentrations of c-Met ligand, we quantified HGF levels in bronchoalveolar lavage samples, which contain secretions from tumor cells as well as resident neutrophils and macrophages. Indeed, HGF levels were higher in samples from $Kras^{LA1}$ mice ($n = 9$) than those of wild-type littermates ($n = 6$; Fig. 1B).

In light of the reported role of c-Met in maintaining the survival of tumor cells as well as other cell types in the microenvironment such as endothelial cells (16), we next determined which cell types within AAH and adenomas expressed c-Met by performing immunohistochemical and immunofluorescence studies. Immunohistochemical analysis of total c-met revealed diffuse expression within the lesions (Fig. 1C, panels a-d), suggesting that c-Met was expressed by multiple cell types. Consistent with this conclusion, dual immunofluorescence studies using antibodies against total c-met and markers for specific cell types, including surfactant protein-C for type II alveolar cells, F4/80 for macrophages, and CD31 for vascular endothelial cells, demonstrated staining in all three cell types (Fig. 1C panels i, n, and s).

Anti-tumor Effects of PHA-665752 in $Kras^{LA1}$ Mice

$Kras^{LA1}$ mice were treated for 6 d with PHA-665752 at high-dose (25 mg/kg/day, $n = 5$) or low-dose (12.5 mg/kg/day, $n = 5$) or vehicle ($n = 5$). The doses of PHA-665752 were chosen based on a previous study that showed dose-dependent growth inhibition of xenograft tumors in nude mice (12). Following the completion of therapy, mice were sacrificed and subjected to autopsy to quantify the numbers of lung lesions on the pleural surfaces. One lung was frozen and the other was formalin-fixed for later use in western blot and immunohistochemical analyses, respectively. For immunohistochemical studies, a tissue microarray was constructed with punch-biopsy samples of all identifiable AAH and adenoma lesions of each $Kras^{LA1}$ mouse treated with PHA-66572 or vehicle.

We found a reduction in the numbers of lesions in mice treated with high-dose but not low-dose PHA-665752 (Fig. 2A), demonstrating dose-dependent anti-tumor effects. We next investigated the lungs for biochemical markers that correlated with the differential effects of PHA-665752 on the numbers of lung lesions at the two doses. We performed immunohistochemical analysis of lung tissues to quantify Ser^{473}-phosphorylated AKT, which activates survival signals in $Kras^{LA1}$ mice (17), and western blotting of whole lung extracts to quantify Tyr^{1234}-phosphorylated c-Met and cleavage of the caspase-3 substrate poly(ADP-ribose)polymerase (PARP), a marker of apoptotic cells. Although the reduction in Tyr^{1234}-phosphorylated c-Met was roughly equivalent in the

low- and high-dose groups (Fig. 2B), the reduction in Ser^{473}-phosphorylated AKT (Fig. 2C) and cleavage of PARP (Fig. 2D) were more prominent in mice treated with high-dose than in those treated with low-dose PHA-665752. Thus, the dose-dependent effects of PHA-665752 on tumor numbers correlated with its effects on biochemical markers of cell survival and apoptosis but not c-Met phosphorylation.

We next examined which cell types within the premalignant lesions underwent apoptosis following high-dose PHA-665752 treatment by performing dual-immunofluorescence assays using cleaved caspase-3 as a marker of apoptotic cells, which was merged with surfactant protein-C, F4/80, and CD31 as markers of tumor cells, macrophages, and vascular endothelial cells, respectively. Cleaved caspase-3 staining overlapped with that of SPC (Fig. 3A panel d) and CD31 (Fig. 3A panel h) but not F4/80 (data not shown), indicating that it was the tumor cells and vascular endothelial cells but not macrophages that had undergone apoptosis. We quantified the percentages of apoptotic cells that were endothelial cells and tumor cells. In tumor sections stained with CC3 and SPC, 84 CC3-positive cells were identified, of which 31 (37%) were SPC-positive. In tumor sections stained with CC3 and CD31, 90 CC3-positive cells were identified, of which 10 (11%) were CD31-positive. Thus, apoptotic tumor cells were more prevalent than apoptotic intra-tumoral endothelial cells. To examine whether the pro-apoptotic effect of PHA-665752 on endothelial cells resulted in a reduction in the numbers of intra-tumoral endothelial cells, we quantified endothelial cells by performing immunohistochemical analysis of Factor vIII staining, which revealed a dose-dependent reduction in endothelial cells associated with PHA-665752 treatment (Fig. 3B). In contrast, the numbers of infiltrating macrophages and neutrophils did not change (data not shown). We therefore conclude that PHA-665752 induced the apoptosis of endothelial cells and tumor cells.

c-Met-dependent and Independent Effects of PHA-665752

Although the mice exhibited equivalent reductions in intra-tumoral c-Met phosphorylation following treatment with low-dose or high-dose PHA-665752, only the high-dose group experienced a reduction in tumor numbers, suggesting that PHA-665752 inhibited tumorigenesis, at least in part, through c-Met-independent mechanisms. To further investigate this possibility, we compared the pro-apoptotic effect of PHA-665752 to that of c-Met siRNA in LKR-13, an adenocarcinoma cell line derived from $Kras^{LA1}$ mice, and MEC, an immortalized murine lung endothelial cell line. These cell lines were chosen as models of the two cell types found to undergo apoptosis in $Kras^{LA1}$ mice treated with PHA-665752.

Treatment of MECs with PHA-665752 caused reductions in c-Met phosphorylation after 4 h (Fig. 4A) and cell densities after 72 h (Fig. 4B), which was due in part to apoptosis as shown by western blot analysis of cleaved caspase-3 (Fig. 4C) and Hoechst staining of cells to detect

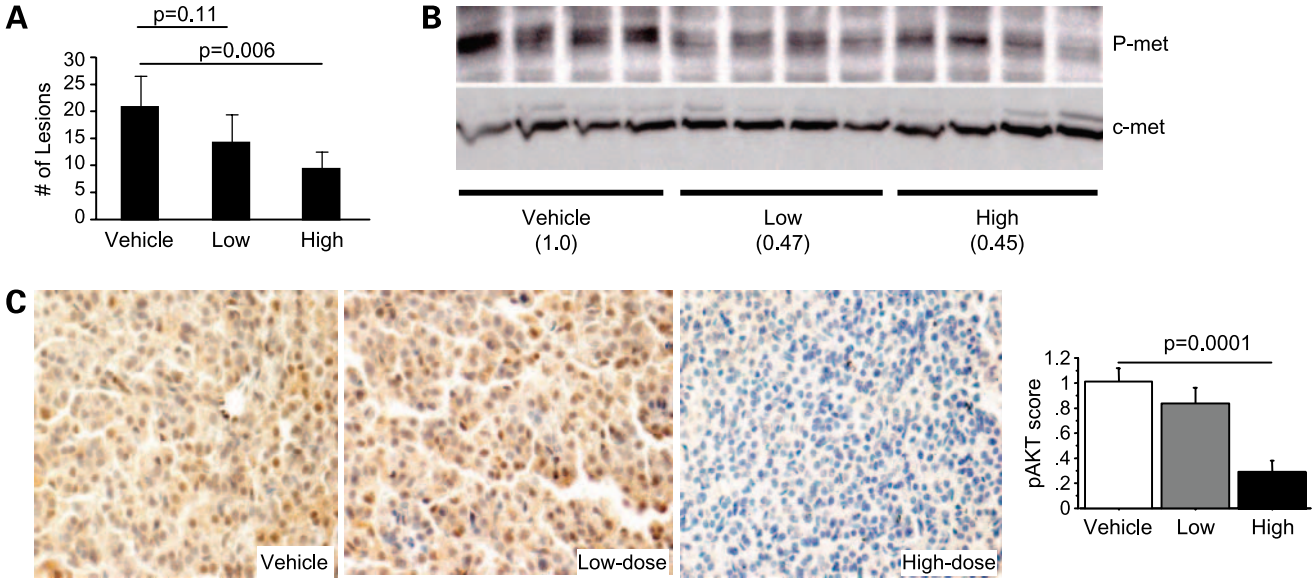

Figure 2. PHA-665752 inhibited c-met signaling and induced apoptosis in the lungs of $Kras^{LA1}$ mice. **A,** PHA-665752 decreased the numbers of premalignant lung lesions. Numbers of lesions on pleural surfaces counted at the time of autopsies of mice in the three treatment groups (mean ± S.D.). **B,** PHA-665752 inhibited c-met phosphorylation. Mice were treated with vehicle, low-dose (low) PHA-665752, or high-dose (high) PHA-665752 (n = 4 for each). Western blot analysis of whole-lung lysates. Mean densitometric values (-fold) of Tyr-1234-phosphorylated c-met (P-met) for each treatment group were determined relative to that of vehicle, which was set at 1.0, after correcting for differences in total c-met. **C,** PHA-665752 decreased AKT phosphorylation. Left, representative images (×10 magnification) of premalignant lung lesions stained for Ser473-phosphorylated AKT by immunohistochemistry. Right, scoring of AKT phosphorylation (mean ± S.D.) for the three treatment groups.

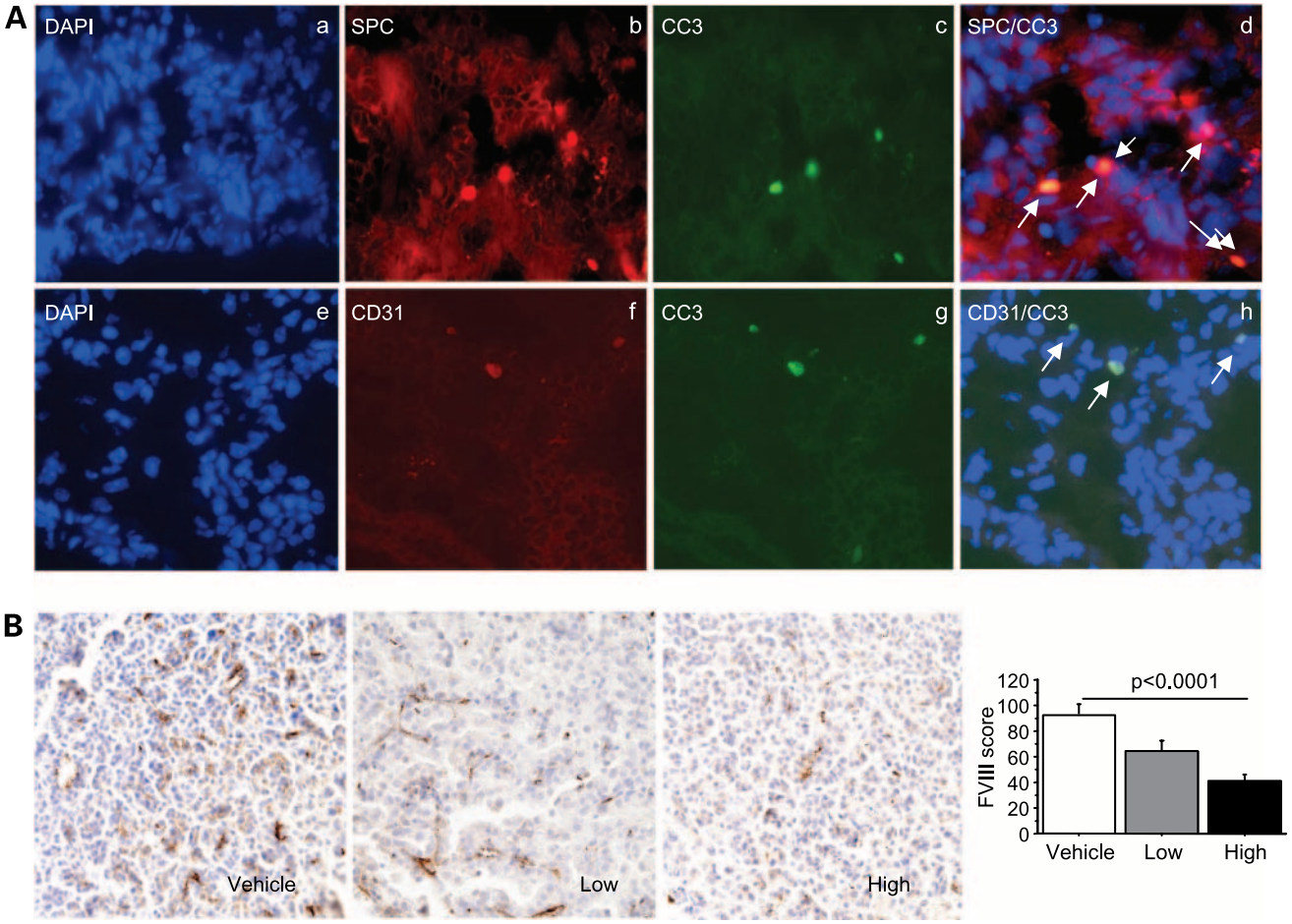

Figure 3. PHA-665752 induced apoptosis of specific cell types within premalignant lung lesions. **A,** PHA-665752 induced apoptosis of tumor cells and vascular endothelial cells. Dual-immunofluoresence staining was performed on lung tissues from mice that received high-dose PHA-665752 using antibodies against cleaved caspase-3 (CC3) (panels c and g) and markers for specific cell types, including SPC for tumor cells (panel b) and CD31 for vascular endothelial cells (panel f). CC3 stains were merged with corresponding stains for SPC (panel d) and CD31 (panel h). DAPI stains (panels a, e) are illustrated. All images are ×40 magnification. **B,** PHA-665752 decreased the numbers of endothelial cells. Left, representative images of premalignant lesions stained immunohistochemically for Factor vIII (×10 magnification). Right, scoring of Factor vIII-positive cells (means ± S.D.) in lung sections from mice in the three treatment groups.

nuclear condensation and fragmentation (data not shown). The effects of PHA-665752 on c-met phosphorylation in MECs were dose-dependent and correlated closely with changes in cell numbers and caspase-3 cleavage, suggesting that these were ''on-target'' effects of PHA-665752 in this cell type. Transient transfection of MECs with *c-Met* siRNA achieved a 50% knock-down in c-Met protein levels after 72 h, which decreased cell numbers by 40% and induced apoptosis as shown by western blotting to detect caspase-3 (Fig. 4D). Together, these findings suggest that c-Met inhibition induced apoptosis of MECs. Treatment of LKR-13 cells with PHA-665752 caused dose-dependent reductions in c-Met phosphorylation after 4 h (Fig. 5A) and cell density after 72 h (Fig. 5B) as well as biochemical (Fig. 5C) and morphologic (data not shown) evidence of apoptosis. However, the IC_{50} dose in LKR-13 cells based on cell density measurements (2.7 μM) was considerably higher than the IC_{50} dose based on reductions in phosphorylated c-Met (0.63 μM), suggesting that cytotoxocity was mediated in part through ''off-target'' effects. Consistent with this conclusion, despite having achieved an efficient (80%) reduction in c-Met protein levels, transient transfection of LKR-13 cells with *c-Met* siRNA caused no change in cell density or biochemical evidence of apoptosis after 72 h (Fig. 5D), indicating that c-Met depletion was not sufficient to induce apoptosis of LKR-13 cells. Collectively, these findings suggest that c-Met inhibition was not sufficient to induce apoptosis of LKR-13 cells and that PHA-665752 mediated its effects through c-Met-dependent and -independent mechanisms.

Discussion

Effective therapeutic approaches are needed for the prevention and treatment of *K-ras*-mutant NSCLC. To address this need, here we investigated the efficacy of

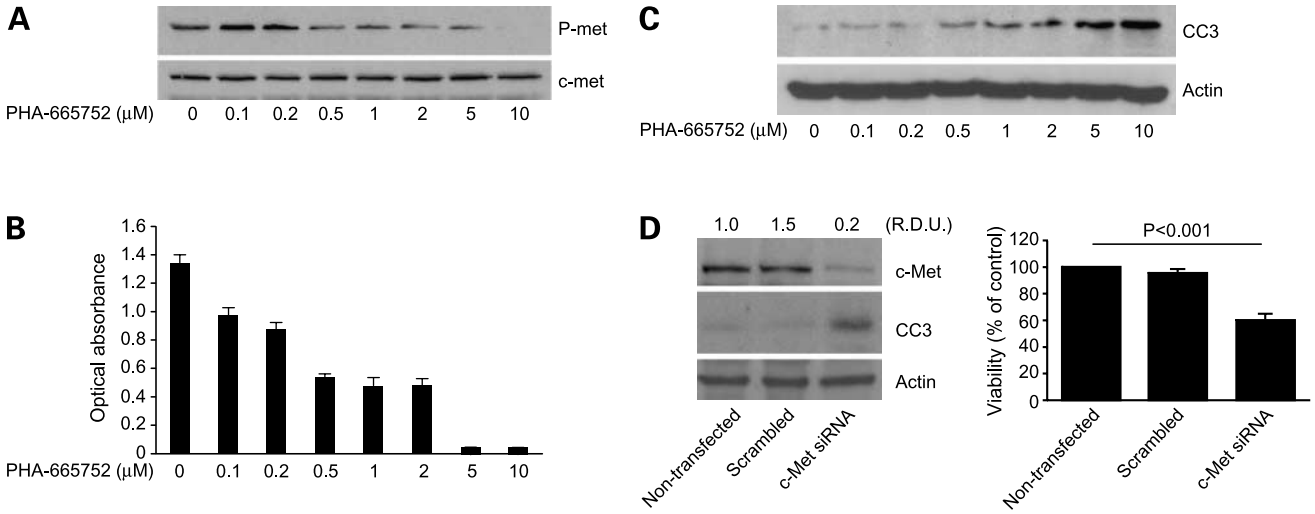

Figure 4. Effects of PHA-665752 or c-Met siRNA on MECs. **A,** PHA-665752 decreased c-met phosphorylation at Tyr-1234. Western blot analysis of Tyr-1234-phosphorylated c-met (P-met) in MECs after 4 h of PHA-665752 treatment. **B,** PHA-665752 decreased cell density. MTT assays were performed on MECs after 72 h of PHA-665752 treatment. **C,** PHA-665752 induced caspase-3 cleavage. Western blot analysis of cleaved caspase-3 (CC3) in MECs after 24 h of PHA-665752 treatment. Actin was used as a loading control. **D,** c-Met depletion induced apoptosis of MECs. MECs were transiently transfected for 72 h with c-Met or scrambled siRNA and subjected to western blot analysis (left panel) or MTT assays (right panel). Quantification of c-Met bands by scanning densitometry is indicated (left panel, top) in relative densitometric units (R.D.U.).

the c-Met inhibitor PHA665752 in $Kras^{LA1}$ mice based on previous reports demonstrating a role for c-Met in malignant progression of Ras-mutant cancer cells (4). We found that the concentration of HGF was increased in bronchoalveolar lavage fluids, c-Met was ubiquitously expressed and highly phosphorylated in lung lesions, and short-term treatment with PHA-665752 reduced the numbers of lung lesions and induced apoptosis of tumor cells and intra-tumoral endothelial cells. We conclude from these findings that c-Met was activated during lung tumorigenesis, and PHA-665752 reversed lung premalignant lesions in this model. Although these findings suggest

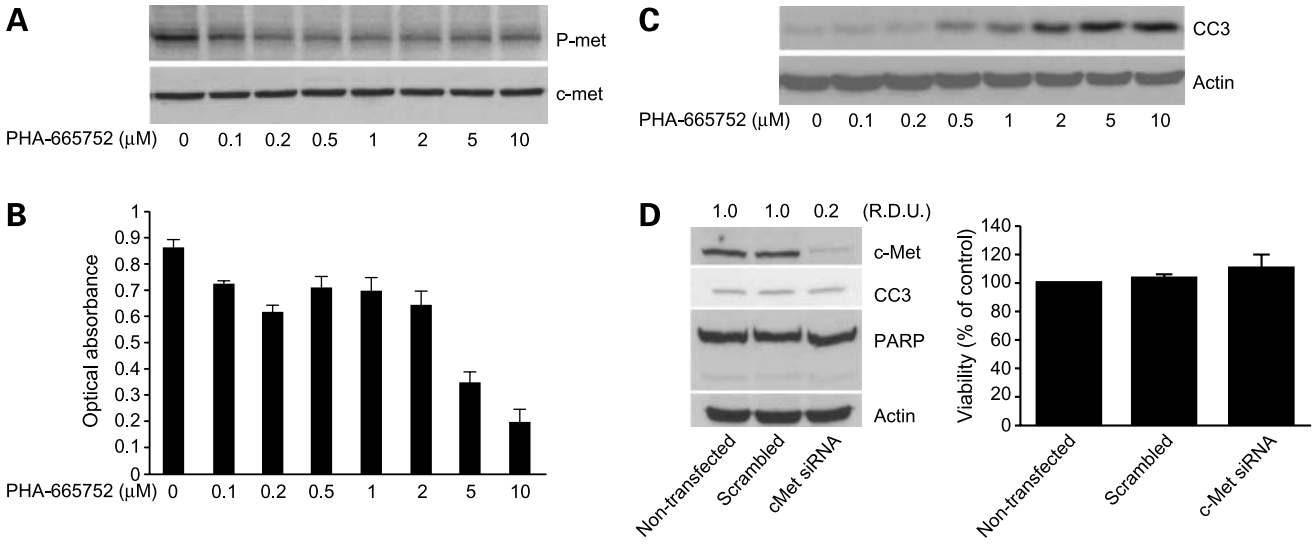

Figure 5. Effects of PHA-665752 or c-Met siRNA on LKR-13 cells. **A,** PHA-665752 decreased c-met phosphorylation at Tyr-1234. Western blot analysis of Tyr-1234-phosphorylated c-met (P-met) in LKR-13 cells after 4 h of PHA-665752 treatment. **B,** PHA-665752 decreased cell density. MTT assays were performed on LKR-13 cells after 72 h of PHA-665752 treatment. **C,** PHA-665752 induced caspase-3 cleavage. Western blot analysis of cleaved caspase-3 (CC3) in LKR-13 cells after 24 h of PHA-665752 treatment. Actin was used as a loading control. **D,** c-Met depletion induced no evidence of apoptosis. LKR-13 cells were transiently transfected for 72 h with c-Met or scrambled siRNA and subjected to western blot analysis (left panel) or MTT assays (right panel). Quantification of c-Met bands by scanning densitometry is indicated (left panel, top) in relative densitometric units (R.D.U.).

that PHA-665752-induced apoptosis had a substantial impact on tumor growth, we have not excluded the possibility that other biologic processes, such as proliferative arrest or necrosis of tumor cells, also contributed to the reduction in tumor numbers in these mice.

Several of the findings presented here argue in favor of c-Met as a therapeutic target in $Kras^{LA1}$ mice, whereas others suggest that c-Met inhibition was not sufficient to inhibit tumor growth in this model. First, low-dose PHA-665752 treatment achieved an inhibition of c-Met phosphorylation that was equivalent to that of high-dose therapy, but low-dose treatment did not cause tumor regression. Second, the PHA-665752 IC_{50} value in LKR-13 cells based on cell density exceeded the IC_{50} value based on c-Met phosphorylation. Third, transfection of LKR-13 cells with *c-Met* siRNA achieved an 80% reduction in c-Met protein but did not decrease cell numbers. Together, these findings suggest that the cytotoxic effects of PHA-665752 against tumor cells in this model were mediated in part through c-Met-independent mechanisms. ''Off-target'' effects of PHA-665752 reported previously include inhibition of the Ron (IC_{50} value 0.9 μM) and Flk-1 (IC_{50} value 2.5 μm) receptor tyrosine kinases (12). Cell lines that are highly dependent upon c-Met for survival due to overexpression of c-Met typically have PHA-665752 IC_{50} values less than 1.0 μM (12, 18), suggesting that tumor cell cytotoxic effects observed at doses higher than 1.0 μM are due in part to c-Met-independent mechanisms.

Our data show that PHA-665752 induced apoptosis of tumor cells and intra-tumoral endothelial cells in mice. Direct effects of PHA-665752 on tumor cells were not likely to have been the primary contributor to its anti-tumor effect in this study because peak serum PHA-665752 levels in mice administered the same dose range of PHA-665752 are approximately 1-3 μM.[8] This concentration was not sufficient to induce apoptosis of LKR-13 cells *in vitro*. Therefore, we favor the conclusion that tumor cell apoptosis was caused in part by PHA-665752-induced endothelial cell death. Although our findings show that endothelial cells were not the predominant intra-tumoral cell type undergoing apoptosis in mice, endothelial cells constitute a minority of the total intra-tumoral cell population, and removal of a small number of endothelial cells could affect a larger number of tumor cells that depend upon a single blood vessel for survival.

The findings presented here corroborate a previous report demonstrating anti-angiogenic effects of PHA-665752 as well as that of another c-Met inhibitor, PF-2341066, and are supported by a large body of evidence demonstrating a fundamental role for c-Met in tumor angiogenesis (16, 19, 20). c-Met promotes angiogenesis by increasing the transcription of angiogenic mediators, including, among others, interleukin-8 (IL-8) and vascular endothelial growth factor (21–24). The murine functional homologues of IL-8 (KC and MIP-2α) are highly expressed in the lungs of $Kras^{LA1}$ mice, and the passive immunization of $Kras^{LA1}$ mice using neutralizing antibodies against CXCR2, a receptor for these chemokines, induces apoptosis in vascular endothelial cells within premalignant lung lesions and inhibits lung tumorigenesis (15). IL-8 is also a direct transcriptional target of mutant *Ras* through its activation of NFκB and mitogen-activated protein kinases (25). Additional studies will be required to identify the role of c-Met as an intermediate in the activation of IL-8 expression by mutant *K-ras*.

Additional research is needed to identify those NSCLC patients most likely to benefit from treatment with c-Met inhibitors. Some recent reports have shed light on this question. Cancer cell lines with *c-Met* genomic amplification are highly sensitive to the cytotoxic effects of PHA-665752 *in vitro* (18). *EGFR*-mutant NSCLC cells that initially respond to treatment with EGFR TKIs subsequently develop resistance to treatment in part through *c-Met* amplification, and combined treatment of these cells with PHA-665752 plus EGFR TKI overcomes this resistance (26). Tumor cell sensitivity to c-Met inhibitors might be enhanced *in vivo* by anti-angiogenic effects of these agents or by cooperative effects with other anti-cancer agents, such as rapamycin (27). In addition to TKIs, other strategies to inhibit c-Met have been developed that might prove equally or more beneficial, including agents that inhibit c-Met at the levels of protein expression (ribozymes), ligand binding (decoy receptors, HGF kringle variant antagonists, or neutralizing antibodies against c-Met or HGF), or kinase activation (small molecule ATP-binding antagonists) have also shown anti-tumor activities in preclinical models (28). These findings constitute ample proof that c-Met should be considered for clinical trials in the treatment of patients with established cancer. Lastly, the response observed here in the $Kras^{LA1}$ early neoplastic disease model indicates that c-Met inhibition may also have a place in the treatment of early lung cancers in humans.

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