Combined MEK and VEGFR Inhibition in orthotopic human lung cancer models results in enhanced inhibition of tumor angiogenesis, growth, and metastasis

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Running title: MEK1/2 and VEGFR inhibition in a mouse model of lung cancer

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ABSTRACT

Purpose: Ras/Raf/MEK/ERK signaling is critical for tumor cell proliferation and survival. Selumetinib is a potent, selective, and orally available MEK1/2 inhibitor. In the current study, we evaluated the therapeutic efficacy of selumetinib alone or with cediranib, an orally available potent inhibitor of all three VEGFR tyrosine kinases, in murine orthotopic NSCLC models.

Experimental Design: NCI-H441 or NCI-H460 KRAS-mutant human NSCLC cells were injected into the lungs of mice. Mice were randomly assigned to treatment with selumetinib, cediranib, paclitaxel, selumetinib plus cediranib, or control. When controls became moribund, all animals were sacrificed and assessed for lung tumor burden and locoregional metastasis. Lung tumors and adjacent normal tissues were subjected to immunohistochemical analyses.

Results: Selumetinib inhibited lung tumor growth and, particularly at higher dose, reduced locoregional metastasis, as did cediranib. Combining selumetinib and cediranib markedly enhanced their antitumor effects, with near complete suppression of metastasis. Immunohistochemistry of tumor tissues revealed that selumetinib alone or with cediranib reduced ERK phosphorylation, angiogenesis, and tumor cell proliferation and increased apoptosis. The antiangiogenic and apoptotic effects were substantially enhanced when the agents were combined. Selumetinib also inhibited lung tumor VEGF production and VEGFR signaling.

Conclusions: In the current study, we evaluated therapy directed against MEK combined with antiangiogenic therapy in distinct orthotopic NSCLC models. MEK inhibition resulted in potent antiangiogenic effects with decreased VEGF expression and
signaling. Combining selumetinib with cediranib enhanced their anti-tumor and antiangiogenic effects. We conclude that combining selumetinib and cediranib represents a promising strategy for the treatment of NSCLC.

**Translational Relevance:** Lung cancer is a major worldwide health problem and a leading cause of cancer-related death and morbidity. The outcome for patients with lung cancer has not significantly changed in over two decades and more effective therapies for lung cancer are urgently needed. We evaluated combined MEK blockade and angiogenesis inhibition directed against VEGFR signaling in orthotopic models of human non-small cell lung cancer (NSCLC) that closely recapitulate clinical patterns of lung cancer progression and allow for the study of the influence of lung microenvironment upon response to therapy. MEK inhibition potentiated the effects of anti-VEGF therapy and independently inhibited lung tumor angiogenesis, VEGF production, and VEGFR signaling. These data provide a strong basis for clinical trials combining selumetinib and cediranib in lung cancer patients.
INTRODUCTION

Lung cancer is a global problem and new therapies and approaches to the treatment of lung cancer are urgently needed (1, 2). The approach to the treatment of non-small cell lung cancer (NSCLC) is currently in transition and an emerging strategy for the treatment of lung and other cancers is personalized therapy based on the molecular characteristics of a tumor from individual patients. As research on molecular targeted therapy and biomarkers that predict the effectiveness of such therapies proceeds, more effective treatment strategies for lung cancer are becoming possible (3). Antiangiogenic therapy and therapies directed against growth factors and their associated signaling pathways have emerged as compelling targets against lung cancer that are now being used in the clinic.

Antiangiogenic therapy, and in particular therapy targeting the VEGF signaling pathway, has shown promise in the treatment of lung cancer and bevacizumab, an antibody directed against vascular endothelial growth factor (VEGF), can improve overall survival when it is combined with carboplatin and paclitaxel for patients with advanced lung cancer (4). However, when bevacizumab was combined with cisplatin and gemcitabine for advanced NSCLC progression free survival was improved but no overall survival advantage was observed (5, 6) nor was there a benefit for overall survival when it was combined with cisplatin or carboplatin and etoposide in patients with extensive-stage small cell lung cancer (SCLC) (7). VEGF signaling remains an important target in anticancer therapy because of its role in angiogenesis (8), which is fundamental to tumor growth and spread (9), but it has become apparent that treatment with bevacizumab alone or with systemic chemotherapy may not be sufficient for the
treatment of some advanced lung cancers. Further, toxicities associated with bevacizumab, when it is used with systemic chemotherapy, have been observed in clinical trials in lung cancer patients (10).

Cediranib (AZD2171) is an orally available and highly potent VEGFR-1, 2, and 3 tyrosine kinase inhibitor with additional activity against the platelet-derived growth factor-β receptor and c-Kit (11-13). Cediranib is currently being investigated in clinical trials both alone and in combination with chemotherapy for a variety of malignancies. The addition of cediranib to standard chemotherapy for metastatic colorectal cancer was associated with improved progression free survival but did not improve overall survival in a Phase III trial (14, 15). In a Phase III trial of cediranib or lomustine alone and in combination in patients with recurrent glioblastoma, no statistically significant difference in median progression-free survival of 92 days for the cediranib arm or 125 days in the combination arm was observed as compared with 82 days for lomustine alone (16, 17). Progression free survival at 6 months was 16% in the cediranib arm which was lower than the 25.8% survival at 6 months reported in the earlier Phase II trial of cediranib for recurrent glioblastoma (18). For lung cancer, a phase II/III trial (BR24) in which cediranib at a 30 mg dose was combined with carboplatin and paclitaxel for advanced NSCLC demonstrated improved response rates and progression-free survival but was terminated early due to concerns of toxicity (19, 20). Although the clinical experience for cediranib in lung and other cancers does demonstrate evidence for activity, the results show that it may be prudent to combine it with other biologically targeted therapeutics for the treatment of lung cancer.
Targeted therapy directed against the epidermal growth factor receptor (EGFR) with gefitinib (21), erlotinib (22), or cetuximab (23) are currently being used in the clinic for the treatment of NSCLC with improvements in progression free and overall survival in subsets of patients (24). EGFR tyrosine kinase inhibitors have shown single agent activity in lung cancer patients whose tumors harbor EGFR mutations (25) but the efficacy of these agents for cancers that harbor Kras mutations is unclear (26). Although some patients will experience a durable benefit from these agents, in most cases the improvement in survival can only be measured in weeks or months. Further, these agents may only be effective in a small percentage of lung cancer patients and resistance to therapy can develop (27).

In order to overcome some of the limitations of EGFR and other growth factor receptor inhibitors and to more broadly target lung cancer growth, therapeutic strategies to target signaling pathways that are downstream of these receptors have been investigated (28). The mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 kinases (MEK) that are situated downstream of Ras and Raf represent attractive therapeutic targets for lung and other cancers. MEK signaling is crucial in the regulation of multiple processes, including tumor cell proliferation and survival, in a variety of cancers, including lung cancer, and its inhibition offers a particularly attractive therapeutic target (29, 30). Selumetinib (AZD6244, ARRY-142886), a potent, selective, and orally available MEK1/2 inhibitor (31, 32), has been studied clinically in a variety of cancers, including lung cancer (33) and is currently being evaluated in a phase II clinical trial in KRAS-mutant NSCLC. However, the inhibition of MEK signaling alone may not
be sufficient in patients with advanced lung cancer and feedback mechanisms in this pathway may be problematic when it is used alone (34).

To determine the efficacy of selumetinib for lung cancer growing in the lung and to investigate its use for the treatment of lung cancer with antiangiogenic therapy, we have studied both monotherapy and combination with targeted therapy directed against VEGFR signaling with cediranib in orthotopic models of human lung cancer. We report the results of this novel combination therapy in our murine orthotopic models of human lung cancer that closely recapitulate the clinical behavior of lung cancer in humans (35) using two different human NSCLC cell lines that harbor mutations in K-Ras.

**MATERIALS AND METHODS**

**Cell cultures**

The human lung adenocarcinoma cell line NCI-H441 and the human large cell lung cancer cell line NCI-H460, both of which have KRAS mutations (36, 37), were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were molecularly characterized by The University of Texas MD Anderson Cancer Center’s Cell Line Characterization Shared Resource and determined to be free of *Mycoplasma* and pathogenic murine viruses. Cells were maintained in RPMI-1640 with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution, and penicillin-streptomycin (Invitrogen, Grand Island, NY) and incubated in an atmosphere of 5% CO₂ and 95% air at 37°C.

**Orthotopic model of human non-small cell lung cancer**
6 – 8 week old male athymic nude mice (Taconic, Hudson, NY) were used for experiments in accordance with current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, the National Institutes of Health, and The University of Texas MD Anderson Cancer Center. Mice were anesthetized with sodium pentobarbital (50 mg/kg body weight) and placed in the right lateral decubitus position. A 5-mm skin incision overlying the left chest wall was made and the left lung was visualized through the pleura. 1x10^6 NCI-H441 cells or 5x10^5 NCI-H460 cells (single-cell suspensions, greater than 90% viability) in 50 μg of growth factor-reduced Matrigel (BD Biosciences) in 50 μL of Hank’s balanced salt solution were injected into the left lungs of the mice through the pleura using a 30-gauge needle. After tumor cell injection, the wound was stapled and the mice were placed in the left lateral decubitus position and observed until fully recovered.

**Drug preparation and treatment schedules**

Selumetinib and cediranib (provided by AstraZeneca, Macclesfield, UK) were formulated in vehicle consisting of either 0.5% w/v hydroxypropyl methyl cellulose/0.1% w/v Tween 80 or 1% w/v polysorbate 80, respectively. Paclitaxel (Bristol-Myers Squibb, New York, NY) was dissolved in saline immediately before use. Fourteen days after the implantation of NCH-H441 cells or 10 days after the implantation of NCI-H460 cells, when the lung tumors were established, mice (10-13 per group) were randomly allocated to receive treatment with selumetinib (12.5 or 25 mg/kg twice daily by oral gavage), cediranib (3 mg/kg once daily by oral gavage), paclitaxel (200 μg/mouse once a week intraperitoneally), selumetinib (25 mg/kg bid by oral gavage) plus cediranib, or
vehicle control. Selumetinib was given twice daily at 8 hour intervals and cediranib was given once daily, 4 hours after the first daily dose of selumetinib. Treatment was continued until the control mice became moribund (55 days for the NCI-H441 model or 19 days for the NCI-H460 model), at which point all mice were killed by CO₂ inhalation and assessed for lung weight, primary lung tumor volume \((\pi \times d_{\text{long}} \times d_{\text{short}}^2 ÷ 6)\), and the presence of mediastinal lymph node disease or distant metastasis. For the NCI-H460 model, in which mice also develop chest wall tumors, the aggregate volume of the chest wall tumors was combined with the primary lung tumor volume to create the total tumor volume.

**Histologic preparation and immunohistochemical-immunofluorescent staining**

Primary lung tumors and adjacent lung tissues were removed from all of the mice in every treatment group and fixed with 10% formalin and embedded in paraffin or directly frozen in OCT cryoembedding compound and then sectioned and stained with hematoxylin and eosin or immunoantibodies. Immunostaining for CD31 (rat anti-mouse, Pharmingen) and dual immunofluorescence staining for CD31 and activated VEGFR2-3 (rabbit anti-human, EMD Biosciences) were performed with frozen tissues as described previously (38). Sections of formalin-fixed, paraffin-embedded tissue specimens were used to assess cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), Ki67 (rabbit a-human, Thermo Scientific), VEGF (goat anti-rat/human, R&D), VEGFR2 (rabbit a-human, Santa Cruz), and phosphorylated MAPK 44/42 (Erk1/2) (Thr202/Tyr204) (anti-human, SignalStain kit from Cell Signaling) as described previously (38-40).
Quantification of microvessel density, vascular area, Ki67, caspase-3, and activated ERK

For quantification of microvessel density and vascular area in lung tumors, up to 4 random fields for each tumor section at x100 magnification (60% center field) were captured after staining with anti-CD31 antibody. Microvessels were counted and vascular area was calculated using Image Pro software (Media Cybernetics, Inc. Bethesda, MD). Microvessel density was presented as the number of microvessels per field and as the percentage of vascular pixel area to field pixel area. The number of Ki67- and activated ERK-positive nuclei was counted regardless of the immunointensity in 4 random fields at x100 magnification (60% center field). The number of cleaved caspase-3–positive cells was counted in similar fashion but at x200 magnification. Ki67 immunoreactivity was expressed as the percentage of Ki67-positive cells to the total tumor cells per field.

H-scoring of VEGF and VEGFR2 immunoreactivity

For semi-quantification of VEGF and VEGFR2 immunoreactivity, H-scores were independently generated by two of the authors (O.T. and A.B.) who were blinded as to treatment group as described previously (41), with slight modification. H-scores were based on findings from up to 4 randomly selected fields for each tumor section at x100 magnification (60% center field). Staining intensity was graded as undetectable (0), weak (1), medium (2), or strong (3) and the percentage of positive cells per field was calculated. The intensity score and the percentage of positive cells were then multiplied to give an H-score (possible range, 0-300).
**Immunofluorescence quantification**

Dual fluorescent staining for endothelial cells (CD31, red), activated VEGFR2/3 (green), and tumor cell nuclei (blue) were completed as described above. The expression of activated VEGFR2/3 in tumor-associated endothelial cells was identified by co-localized yellow fluorescence. The pixel areas of green, blue, red, and yellow were quantified using Image Pro Plus (Media Cybernetics, Inc. Bethesda, MD) in up to 4 random fields for each tumor section at x200 magnification. Quantification of total activated VEGFR2/3 expression was presented as an index of green area to blue area. Activated VEGFR2/3 expression in endothelial cells was presented as an index of yellow area to red area. All of the quantification data were presented as means ± standard error of the means (SEM).

**Statistical analysis**

Data were analyzed by using Prism5 software (GraphPad Software, Inc., La Jolla, CA). To analyze immunohistochemical and dual-fluorescent findings, the mean value for each tumor was first calculated from captured fields. The Kruskal-Wallis test followed by the Mann-Whitney U test were then used to assess differences among the treatment and control groups with respect to body weight, tumor volume, left lung weight, and quantitative immunohistochemical and dual-fluorescent findings within treatment groups and between treatment and control groups. **We used the Kruskal-Wallis as a gate-keeping procedure (i.e. a protected testing procedure) such that we would not do any pair-wise comparisons unless the Kruskal-Wallis test was significant.**
This procedure controls the experiment-wise error rate against making at least one false pair-wise inference in the case that the experiment-wise null is true. In addition to this protected procedure, because multiple comparisons were anticipated but use of the Bonferroni correction for all interesting pairwise comparisons would have resulted in too stringent a p value, we considered a p value of less than 0.007 as statistically significant to account for the multiple comparisons. Using a comparison-wise type 1 error rate of 0.007 controls the experiment-wise type 1 error rate at 0.10. Differences in the incidence of lymph node metastasis or distant metastasis were analyzed by Fisher’s exact probability tests, and a p value of less than 0.05 was considered statistically significant.

RESULTS

Selumetinib and cediranib block orthotopic human lung cancer progression in the lung and thorax

To evaluate the therapeutic efficacy of selumetinib, alone and in combination with cediranib, we used orthotopic models of lung cancer with NCI-H441 adenocarcinoma or NCI-H460 large cell human NSCLC cells in nude mice. All treatments were well tolerated, with no significant differences among groups in body weight. The incidence of tumor formation was 100% after implantation in the left lung for both models (Tables 1 and 2).

In the NCI-H441 human lung adenocarcinoma model (Figure 1A and Table 1), lung tumors grew within the left lung and spread within the lung and then to the mediastinum. Treatment with selumetinib at both dose levels (12.5 and 25 mg/kg BID)
inhibited the growth of primary lung tumors by 71% and 82%, respectively, compared with controls. Selumetinib, particularly at the higher dose, was also effective in reducing the incidence of mediastinal adenopathy. Cediranib monotherapy also inhibited primary lung tumor growth by 68% and the incidence of mediastinal adenopathy. The anti-tumor and anti-metastatic effects of each agent were substantially enhanced when they were combined with a 90% reduction in median primary lung tumor volume, 73% reduction in median left lung weight, and near complete suppression mediastinal lymph node metastasis. Treatment with paclitaxel reduced lung primary lung tumor volume by 74% but with only modest effects upon mediastinal adenopathy that were not statistically significant.

Similar results were observed in the NCI-H460 human large cell lung cancer orthotopic model (Fig. 1B, Table 2). In the NCI-H460 model, lung tumors grew within the left lung and spread within the lung and then to the mediastinum and also to chest wall of the left hemi-thorax. Paclitaxel treatment was only marginally effective in the NCI-H460 model, as compared to the NCI-H441 model. Selumetinib, at the lower dose, reduced primary lung tumor volume by 65% and the total tumor volume by 71%, as compared with control, but did not substantially reduce the incidence of mediastinal lymph node metastasis. At the higher dose, selumetinib reduced primary lung tumor volume by 90%, total tumor volume by 92% and decreased the incidence of mediastinal lymph node metastasis. Cediranib monotherapy was also efficacious and reduced primary tumor volume by 78% and total tumor volume by 84%, but had only modest effect upon mediastinal lymph node metastasis, whereas distant metastasis was completely inhibited. The anti-tumor and anti-metastatic effects of each agent were
substantially enhanced when selumetinib and cediranib were combined with a reduction in primary lung tumor volume by 96% and total lung tumor volume by 97% and the near complete suppression of lymph node metastasis.

**Selumetinib and cediranib inhibit tumor cell proliferation and increases tumor cell apoptosis in lung tumors**

To characterize the mechanism of tumor growth inhibition observed in both of our lung cancer models by selumetinib and cediranib, lung tumors were subjected to immuhistochemical analyses. Lung tumors from each of the different treatment groups and for each of the 2 lung cancer models were assessed for evidence of tumor cell apoptosis, as determined by staining for cleaved caspase-3 (Figure 2A). Treatment with paclitaxel marginally increased tumor cell apoptosis in both models. Apoptosis was significantly increased by selumetinib in a dose-dependent fashion in the NCI-H441 (p < 0.001) and in the NCI-H460 model (p < 0.007) with an approximate 6 and 3 fold increase, respectively, at the higher dose. Cediranib treatment was also associated with a significant (p < 0.001) increase in lung tumor cell apoptosis, relative to control. The combination of selumetinib and cediranib resulted in a further increase in tumor cell apoptosis with an 8-fold increase in the NCI-H441 model (p < 0.001) and a 5-fold increase (p < 0.001) in the NCI-H460 model.

Tumor cell proliferation for lung tumors from each of the treatment groups in each of the lung cancer models was assessed by evaluating Ki67 expression using immunohistochemistry (Figure 2B). Paclitaxel had virtually no effect upon lung tumor proliferation in the NCI-H441 model and only marginally impacted proliferation in the
NCI-H60 model. Selumetinib monotherapy significantly inhibited lung tumor proliferation in a dose-dependent manner in both lung cancer models with a more pronounced anti-proliferative effect in the NCI-H460 model. Cediranib monotherapy also significantly inhibited lung tumor proliferation in both models with a more pronounced effect upon NCI-H441 tumors. However, when cediranib and selumetinib were combined, there was little evidence for enhancement of their independent anti-proliferative effects examined by pharmacodynamic markers used in these studies.

These data show that the anti-tumor effects of cediranib and selumetinib in our lung cancer models are mediated through both increased tumor cell apoptosis and decreased tumor cell proliferation but that the enhanced anti-tumor activity of the combination of these agents is mediated primarily through increased tumor cell apoptosis.

**Selumetinib inhibits lung tumor ERK activation**

In order to assess the effects of treatment upon MEK signaling in lung tumors, lung tumor tissues were assessed for ERK activation using immunohistochemistry (Figure 3). Both NCI-H441 lung adenocarcinoma and NCI-H460 large cell lung tumors constitutively expressed and activated ERK (pERK). A 2-fold increase in pERK was observed in the NCI-H460 tumors, as compared to the NCI-H441 lung tumors. Treatment with cediranib partially offset ERK activation for lung tumors in both models with a more pronounced in the NCI-H441 model that may be related to the expression of VEGFR2 in lung tumor cells that we have reported previously (42). Treatment with selumetinib resulted in a dose-dependent inhibition of ERK activation for lung tumors in
both lung cancer models. At the higher dose of selumetinib, both alone and in combination with cediranib, the activation of ERK in lung tumors was almost completely suppressed in the NCI-H441 and NCI-H460 lung cancer models. At the lower dose, treatment with selumetinib reduced pERK expression in both lung cancer models but to a lesser degree in the NCI-H460 model than in the NCI-H441 model. These data show that selumetinib treatment can block ERK activation in lung tumors growing orthotopically but that its effects, particularly at lower dose, vary in different lung tumor models.

**Selumetinib inhibits lung tumor angiogenesis with enhanced antiangiogenic effects when combined with cediranib**

To assess the impact of treatment with selumetinib and cediranib alone and in combination for lung cancers growing orthotopically on vasculature and angiogenesis, lung tumors were stained for CD31 and microvessel density and vascular area were then determined (Figure 4). Treatment with paclitaxel had only a modest effect upon lung tumor angiogenesis which was somewhat more pronounced in the NCI-H460 model. Cediranib therapy significantly inhibited lung tumor angiogenesis in both lung cancer models. Selumetinib monotherapy significantly inhibited lung tumor angiogenesis in both lung cancer models with reduced microvessel density and vascular area. The antiangiogenic effects selumetinib and cediranib were markedly increased when they were combined.
Selumetinib, but not cediranib, suppresses the expression of VEGF in orthotopic lung tumors

To clarify the nature of the antiangiogenic effects of treatment (Figure 4), we next evaluated the expression of VEGF (Figure 5A) and its receptor VEGFR2 (Figure 5B) in lung tumors in both of the orthotopic lung cancer models. Treatment with paclitaxel did not impact the expression of VEGF or VEGFR2 in either lung cancer model. Selumetinib monotherapy reduced the expression of VEGF in a dose dependent fashion for the NCI-H441 lung tumors with a 42% decrease at the lower dose (p < 0.0001) and a 62% decrease at the high dose (p < 0.0001), relative to control. In the NCI-H460 lung cancer model, VEGF expression was also offset in lung tumors after treatment with selumetinib with a reduction of VEGF expression of between 20 and 25% as compared to control (p < 0.007). Cediranib treatment did not affect lung tumor VEGF expression in either of the lung cancer models. None of the treatment conditions affected expression of VEGFR2 within the tumor vasculature regardless of which lung cancer cell line was used. These data demonstrate that treatment with the MEK inhibitor selumetinib can offset VEGF expression in 2 distinct orthotopic lung cancer models and suggest that the antiangiogenic effects of treatment with selumetinib in these models is in part due to this decreased expression.

Selumetinib and cediranib inhibit activation of VEGFR2/3 in NCI-H441 and NCI-H460 primary lung tumors and their tumor-associated endothelium

As outlined above, MEK inhibition by selumetinib results in the suppression of angiogenesis in orthotopic NCI-H441 lung adenocarcinomas and NCI-H460 large cell
lung cancers that is due, at least in part, to a decreased VEGF expression in lung tumors. To further elucidate the effects of treatment upon VEGF dependent lung tumor angiogenesis, we characterized VEGFR signaling in both tumor cells and in the tumor vasculature in lung tumor specimens using dual immunofluorescent staining (Figure 6). Paclitaxel treatment had little or no effect upon VEGFR signaling in lung tumor cell or tumor-associated endothelial cells in either lung cancer model. Cediranib treatment blocked VEGFR signaling in both lung tumor cells and tumor-associated endothelial cells in both lung cancer models. Treatment with selumetinib significantly inhibited VEGFR activation in lung tumor cells and tumor-associated endothelial cells in a dose dependent fashion in both the NCI-H441 and NCI-H460 lung cancer models. The most profound effects upon VEGFR activation in lung tumors and their associated vasculatures were observed when selumetinib and cediranib were combined in both lung cancer models. These data show that MEK inhibition by selumetinib results in a decrease in VEGFR activation in lung tumors that is associated with an antiangiogenic effect in lung tumors in 2 distinct lung cancer models.

DISCUSSION

The outcome for patients with advanced lung cancer has not changed substantially over the past several years but recent advances demonstrate that novel biologically targeted therapies can improve the outcomes for subsets of lung cancer patients. However, it has also become apparent the individual agents will need to be combined if the outcomes for lung cancers are to be more broadly improved. In our current studies, we used orthotopic models of human lung adenocarcinoma and large
cell lung cancer that closely mimic clinical patterns of lung cancer spread and progression to investigate antiangiogenic therapy directed against VEGFR signaling with cediranib and molecularly targeted therapy directed against MEK signaling with selumetinib alone and in combination. To our knowledge, this is the first report of the effects of MEK inhibition with antiangiogenic therapy in murine orthotopic models of NSCLC. We found that each agent was effective for the treatment of lung cancer in these models with inhibition of lung tumor growth and, to a lesser degree, lymph node metastasis with efficacy superior to that observed for chemotherapy with paclitaxel. When selumetinib and cediranib were combined, a substantial enhancement of their individual anti-tumor effects was observed with improved efficacy within the lung and a near complete suppression of lung cancer progression and metastasis in both models. Our finding that the combination of these agents impacted both primary tumor and metastatic growth most effectively has direct clinical relevance. Surprisingly, MEK inhibition by selumetinib also suppressed lung tumor angiogenesis and targeted both VEGF production and VEGFR activation in lung tumors, resulting in substantial antiangiogenic effects.

MEK is an attractive therapeutic target for lung cancer treatment because it is situated downstream of Ras and Raf, which are highly activated in Kras-mutated lung cancer (43). Many Kras-mutant cancer cells have been shown to be sensitive to MEK inhibitors (44) and Kras mutations can be detected in up to 30% of lung cancers, dependent upon histology and ethnicity (45, 46), suggesting that a subset of lung cancers would likely be highly sensitive to selumetinib. Our finding that selumetinib was effective in 2 distinct Kras mutant human lung cancer models supports and validates
this hypothesis. Although monotherapy with selumetinib resulted in anti-tumor and some anti-metastatic effects in both of our lung cancer models, the anti-metastatic effects were more apparent in the NCI-H441 lung adenocarcinoma model. The increased anti-metastatic efficacy observed in this model is associated with differences in the constitutive expression and activation of ERK in NCI-H460 and NCI-H441 lung tumors. Both cell lines have KRAS mutations with activation of ERK for lung tumors from both cell lines. However, activated ERK was nearly twice as high in NCI-H460 lung tumors, as compared to NCI-H441 lung tumors, and NCI-460 calls are PI3KCa and LKB1 mutant, both of which might provide a degree of resistance to MEK inhibition. In our studies, a lower dose of selumetinib inhibited ERK activation almost completely in the NCI-H441 model but by only 46 % in the NCI-H460 cells. These findings underscore the importance of MEK signaling in lung cancer progression.

However, additional studies are needed to determine if molecular profiling of lung cancer specimens could be of use to select patients who might best benefit from therapy with selumetinib and to help tailor the dosing of this agent.

Selumetinib was a potent inhibitor of lung tumor angiogenesis in our orthotopic models and the addition of selumetinib to cediranib resulted in a marked enhancement of their individual antiangiogenic effects. Interestingly, selumetinib reduced the production of VEGF in the lung tumors, particularly in the NCI-H441 model. The finding that MEK inhibits VEGF expression is consistent with studies demonstrating that VEGF expression is down-regulated after EGFR inhibition (47) and provides additional mechanism for this process. In vitro studies using head and neck cancer cell lines demonstrate that the VEGF expression after EGFR activation is dependent upon both
PI3K and MAPK signaling (48, 49). The MEK inhibitor PD0325901 decreased the expression of the proangiogenic factors VEGF and interleukin 8 in vitro in human melanoma cells (50). Prior studies in a murine model of hepatocellular carcinoma demonstrated that the anti-tumor and antiangiogenic effects of rapamycin (51) or sorafenib (52) could be enhanced by the addition of selumetinib and that the combination of these agents was associated with modest inhibition in VEGFR signaling in liver tumor lysates with reduced circulating levels of VEGF (51). In pancreatic cancer subcutaneous xenograft murine models, MEK inhibition by selumetinib, but not rapamycin, resulted in decreased microvessel density in the subcutaneous tumors and decreased VEGF levels in tumor lysates (53). Tumor lysates from Calu-6 lung cancer intradermal xenografts in mice treated selumetinib also demonstrated decreases in VEGF levels (54). From these reports and the findings of the present study, we surmise that selumetinib exerts an antiangiogenic effect in lung tumors by directly and indirectly targeting VEGF and its receptors.

The down-regulation of VEGF expression that we observed in NCI-H441 lung adenocarcinomas after therapy with selumetinib was associated with an inhibition of VEGFR signaling both in lung tumor cells and the associated lung tumor vasculature and a resultant antiangiogenic effect. We also observed a potent inhibition of lung tumor angiogenesis and inhibition of VEGFR signaling in lung tumor cells and the associated tumor vasculature in the NCI-H460 large cell lung cancer model. However, in the NCI-H60 model the expression of VEGF after treatment with selumetinib was not as dramatic in NCI-H441 model. These data suggest that the down-regulation of VEGF alone after treatment with selumetinib cannot fully explain the observed antiangiogenic
effects and that the inhibition of MEK by selumetinib may have both direct and indirect effects upon VEGFR signaling with a resultant multicentric antiangiogenic effect. Prior studies in subcutaneous tumor xenograft and in vitro organotypic angiogenesis assays have shown that the expression of dominant-negative MEK1 in the tumor vasculature results was associated with anti-vascular effects and that ERK-MAPK signaling promotes endothelial cell survival sprouting with downregulation of Rho-kinase activity (55). Further investigation is needed to clarify the mechanism by which selumetinib inhibits angiogenesis but our data show that MEK inhibition targets tumor angiogenesis with a multicentric effect.

In summary, our study is, to our knowledge, the first evaluation of therapy directed against MEK in combination with anti-VEGF therapy in orthotopic models of NSCLC. MEK inhibition resulted in potent antiangiogenic effects for lung cancers mediated by down-regulation of VEGF expression and impaired VEGFR signaling. We have further demonstrated that selumetinib or cediranib can significantly inhibit tumor angiogenesis and lung cancer growth and progression with increased tumor cell apoptosis in our orthotopic models. Combining selumetinib with cediranib enhanced their anti-tumor and antiangiogenic effects, with near-complete suppression of lung tumor growth and metastasis. We conclude from these findings that the combination of selumetinib and cediranib represents a promising strategy for the treatment of NSCLC and provides a strong basis for the design of clinical trials for this purpose.

REFERENCES


ACKNOWLEDGMENTS

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<td>33.1 (21.8-36.8)</td>
<td>32 (26.3-34.5)</td>
<td>31.6 (30.2-36.2)</td>
<td>31.9 (29.5-38.5)</td>
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</tr>
<tr>
<td>Tumor incidence</td>
<td>13/13</td>
<td>9/9</td>
<td>10/10</td>
<td>11/11</td>
<td>10/10</td>
<td>11/11</td>
<td></td>
</tr>
<tr>
<td>Left lung weight, mg</td>
<td>560 (300-710)</td>
<td>390 (50-990)</td>
<td>335 (100-710)</td>
<td>280 (100-490)</td>
<td>245 (90-610)</td>
<td>150 (90-280)</td>
<td></td>
</tr>
<tr>
<td>Total lung weight, mg</td>
<td>750 (510-900)</td>
<td>580 (220-1140)</td>
<td>515 (290-910)</td>
<td>480 (290-620)</td>
<td>450 (250-800)</td>
<td>330 (250-480)</td>
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<tr>
<td>Left lung tumor volume, mm³</td>
<td>905 (255-1077)</td>
<td>235 (24-1416)</td>
<td>262 (123-628)</td>
<td>160 (67-628)</td>
<td>294 (14-760)</td>
<td>94 (10-193)</td>
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<tr>
<td>Mediastinal adenopathy</td>
<td>10/13</td>
<td>5/9</td>
<td>5/10</td>
<td>3/11</td>
<td>4/10</td>
<td>0/11</td>
<td></td>
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</tbody>
</table>

Data are presented as medians and ranges except for incidence. \( a p < 0.007, \ b p < 0.001, \ c p < 0.05 \) versus vehicle.
Table 2. Inhibition of tumor growth and metastasis by selumetinib and cediranib in an orthotopic model of lung cancer: NCI-H460 human large cell lung cancer model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment Group</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle (Only)</td>
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<tr>
<td>Body weight, g (range)</td>
<td>29.9 (24.3-32.2)</td>
</tr>
<tr>
<td>Tumor incidence</td>
<td>10/10</td>
</tr>
<tr>
<td>Left lung weight, mg</td>
<td>410 (310-510)</td>
</tr>
<tr>
<td>Left lung tumor volume, mm³</td>
<td>379 (124-644)</td>
</tr>
<tr>
<td>Total tumor volume, mm³</td>
<td>805 (323-1649)</td>
</tr>
<tr>
<td>Mediastinal nodal metastasis</td>
<td>10/10</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>7/10</td>
</tr>
</tbody>
</table>

Data are presented as medians and ranges except for incidence. <sup>a</sup> p < 0.001, <sup>b</sup> p < 0.007, <sup>c</sup> p < 0.05, <sup>d</sup> p < 0.005 versus vehicle.
Figure legends

Figure 1. Anti-tumor effects of selumetinib, cediranib, and paclitaxel in orthotopic models of human NSCLC in mice. *p < 0.007 or **p < 0.001 versus vehicle control or between groups as indicated (Kruskal-Wallis followed by Mann-Whitney U tests).

A. Primary tumors in the left lungs of representative mice from each treatment group after implantation of NCI-H441 human lung adenocarcinoma cells (circled), with mean primary tumor lung volumes and left lung weights. Bars indicate standard error of the mean.

B. Primary tumors in the left lungs (circled) and chest walls of representative mice from each treatment group after implantation of NCI-H460 human lung large cell cancer cells, with mean total tumor volumes (primary lung tumor + chest wall tumors) and left lung weights. Bars indicate standard error of the mean. The dotted horizontal line indicates the normal left lung weight.

Figure 2. Apoptotic and anti-proliferative effects of selumetinib, cediranib, and paclitaxel in orthotopic models of human NSCLC in mice. Lung tumors were collected 2 hours after the last dose of selumetinib, 20 hours after the last dose of cediranib, and 6 days after the last dose of paclitaxel. Quantitative values were determined in four random fields for each tumor. Data are presented as means ± SEM for 9–13 samples per group. The scale bar indicates 100 µm. *p < 0.007, **p < 0.001 versus vehicle control or between groups as indicated (Kruskal-Wallis followed by Mann-Whitney U tests).

A. Immunohistochemical analysis of cleaved caspase 3 in tumors from mice implanted with NCI-H441 or NCI-H460 cells. Representative cleaved caspase-3 staining (brown
staining in positive cells) in lung tumors viewed at x200 magnification. Tumor cell apoptosis (number of cleaved caspase-3 positive cells/field) is shown.

B. Immunohistochemical analysis of Ki67 staining in tumors from mice implanted with NCI-H441 or NCI-H460 cells. Representative Ki67 staining of lung tumors (brown) viewed at x100 magnification, 60% center field. Tumor proliferation (percentage of Ki67-positive cells/total tumor cells) is shown.

**Figure 3.** Effects of selumetinib, cediranib, and paclitaxel upon ERK signaling in orthotopic models of human NSCLC in mice. Representative pERK staining of lung tumors (purple) and quantified pERK expression (number of pERK positive cells/field). All stains are viewed at x100 magnification, 60% center field. Lung tumors were collected 2 hours after the last dose of selumetinib, 20 hours after the last dose of cediranib, and 6 days after the last dose of paclitaxel. Quantitative values were determined in four random fields for each tumor. Data are presented as means ± SEM for 9–13 samples per group. The scale bar indicates 100 µm. *p < 0.007, **p < 0.001 versus vehicle control or between groups as indicated (Kruskal-Wallis followed by Mann-Whitney U tests).

**Figure 4** Antiangiogenic effects of selumetinib, cediranib, and paclitaxel in orthotopic models of human NSCLC in mice as assessed by immunohistochemical analysis of CD31. Representative CD31 staining of lung tumors (brown) viewed at x100 magnification, 60% center field, and microvessel density (number of CD31-positive objects/field) and vascular area (area of CD31-positive objects/field area x 100%) are
shown. Lung tumors were collected 2 hours after the last dose of selumetinib, 20 hours after the last dose of cediranib, and 6 days after the last dose of paclitaxel. Quantitative values were determined in four random fields for each tumor. Data are presented as means ± SEM for 9–13 samples per group. The scale bar indicates 100 µm. *p < 0.007, **p < 0.001 versus vehicle control or between groups as indicated (Kruskal-Wallis followed by Mann-Whitney U tests).

**Figure 5** Effects of selumetinib, cediranib, and paclitaxel upon VEGF and VEGFR expression in orthotopic models of human NSCLC in mice. Lung tumors were collected 2 hours after the last dose of selumetinib, 20 hours after the last dose of cediranib, and 6 days after the last dose of paclitaxel. Quantitative values were determined in four random fields for each tumor. Data are presented as means ± SEM for 9–13 samples per group. The scale bar indicates 100 µm. *p < 0.007, **p < 0.001 versus vehicle control or between groups as indicated (Kruskal-Wallis followed by Mann-Whitney U tests).

A. Immunohistochemical analysis of VEGF in lung tumors from mice implanted with either NCI-H441 or NCI-H460 cells. Representative VEGF staining (brown cytoplasmic staining) in lung tumors and H scores for VEGF expression are shown.

B. Immunohistochemical analysis of VEGFR2 in lung tumors from mice implanted with either NCI-H441 or NCI-H460 cells. Representative VEGFR2 staining (brown cytoplasmic staining) in lung tumors and H scores for VEGFR2 expression are shown.

Figure 6. Effects of selumetinib, cediranib, and paclitaxel upon VEGFR signaling in
orthotopic models of human NSCLC in mice. CD31/pVEGFR2/3 dual fluorescent staining in tumors from mice implanted with either NCI-H441 or NCI-H460 cells was completed. Representative co-localized CD31/pVEGFR2/3 staining of lung tumors viewed at x200 magnification are shown. Fluorescent red indicates CD31-positive endothelial cells; fluorescent green, total pVEGFR2/3-positive cells; fluorescent yellow, pVEGFR2/3-positive endothelial cells. Quantification of total activated VEGFR2/3 expression is presented as an index of green area to blue area. Activated VEGFR2/3 expression in endothelial cells is presented as an index of yellow area to red area. All quantification data are presented as means ± SEM for 9–13 samples per group. The scale bar indicates 50 µm. *p < 0.007 or **p < 0.001 versus vehicle control or between groups as indicated (Kruskal-Wallis followed by Mann-Whitney U tests).
Figure 1A

Left lung tumor volume (mm³)

Vehicle
Selumetinib 12.5 mg/kg BID
Paclitaxel
Selumetinib 25 mg/kg BID
Cediranib 3 mg/kg/day
Combination

**

Left lung weight (mg)

Vehicle
Selumetinib 12.5 mg/kg BID
Paclitaxel
Selumetinib 25 mg/kg BID
Cediranib 3 mg/kg/day
Combination

**

NCI-H441
Figure 1B

- Vehicle
- Paclitaxel 12.5 mg/kg/BID
- Selumetinib 25 mg/kg/BID
- Cediranib 3 mg/kg/day
- Selumetinib (25 mg/kg/BID) and cediranib

Heart
Tumor

**Research.**

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Figure 2A

A Vehicle Paclitaxel Selumetinib 12.5 mg/kg/BID Selumetinib 25 mg/kg/BID Cediranib 3 mg/kg/day Selumetinib (25 mg/kg/BID) and cediranib

**Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.**

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Fig. 2B

<table>
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<th>Treatment</th>
<th>NCI-H441</th>
<th>NCI-H460</th>
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<tbody>
<tr>
<td>Vehicle</td>
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<tr>
<td>Paclitaxel 12.5 mg/kg/BID</td>
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<tr>
<td>Selumetinib 25 mg/kg/BID</td>
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<tr>
<td>Cediranib 3 mg/kg/day</td>
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<tr>
<td>Selumetinib (25 mg/kg/BID)</td>
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<td></td>
</tr>
</tbody>
</table>

**K67 positive nuclear (%)**

- Vehicle
- Paclitaxel
- Selumetinib 12.5 mg/kg/BID
- Selumetinib 25 mg/kg/BID
- Cediranib 3 mg/kg/day
- Selumetinib (25 mg/kg/BID) and cediranib

**Notes:**
- **P** for statistical significance.
- 100 μm scale bar.
Figure 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NCI-H441</th>
<th>NCI-H460</th>
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<td>Vehicle</td>
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<tr>
<td>Paclitaxel</td>
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<tr>
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<td>Selumetinib 25 mg/kg/BID</td>
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<tr>
<td>Cediranib 3 mg/kg/day</td>
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<tr>
<td>Selumetinib (25 mg/kg/BID) and cediranib</td>
<td><img src="combined.jpg" alt="Image" /></td>
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</tbody>
</table>

**Notes:**
- **H441**
  - Positive nuclear #/field: Vehicle > Paclitaxel > Selumetinib 12.5 mg/kg BID > Selumetinib 25 mg/kg BID > Cediranib 3 mg/kg/day > Combination
  - **H460**
  - Positive nuclear #/field: Vehicle > Paclitaxel > Selumetinib 12.5 mg/kg BID > Selumetinib 25 mg/kg BID > Cediranib 3 mg/kg/day > Combination

**Figure Legends:**
- **H441**
  - Positive nuclear #/field: Vehicle > Paclitaxel > Selumetinib 12.5 mg/kg BID > Selumetinib 25 mg/kg BID > Cediranib 3 mg/kg/day > Combination
- **H460**
  - Positive nuclear #/field: Vehicle > Paclitaxel > Selumetinib 12.5 mg/kg BID > Selumetinib 25 mg/kg BID > Cediranib 3 mg/kg/day > Combination

**Figure Notes:**
- **H441**
  - Positive nuclear #/field: Vehicle > Paclitaxel > Selumetinib 12.5 mg/kg BID > Selumetinib 25 mg/kg BID > Cediranib 3 mg/kg/day > Combination
- **H460**
  - Positive nuclear #/field: Vehicle > Paclitaxel > Selumetinib 12.5 mg/kg BID > Selumetinib 25 mg/kg BID > Cediranib 3 mg/kg/day > Combination
Figure 4

A  Vehicle  Paclitaxel  Selumetinib 12.5 mg/kg/BID  Selumetinib 25 mg/kg/BID  Cediranib 3 mg/kg/day  Selumetinib (25 mg/kg/BID) and cediranib

NCI-H441

NCI-H460

100 μm

[Graphs showing microvessel density and vessel area for NCI-H441 and NCI-H460 under different treatments.]
Figure 5A

<table>
<thead>
<tr>
<th>B</th>
<th>Vehicle</th>
<th>Paclitaxel</th>
<th>Selumetinib 12.5 mg/kg/BID</th>
<th>Selumetinib 25 mg/kg/BID</th>
<th>Cediranib 3 mg/kg/day</th>
<th>Selumetinib (25 mg/kg/BID) and cediranib</th>
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<td>NCI-H460</td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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</tbody>
</table>

NCI-H441

- **VEGF H score**
- Vehicle: 150 ± 10
- Paclitaxel: 230 ± 20
- Selumetinib 12.5 mg/kg bid: 250 ± 20
- Cediranib 3 mg/kg/day: 220 ± 20
- Combination: 260 ± 20

NCI-H460

- **VEGF H score**
- Vehicle: 200 ± 10
- Paclitaxel: 270 ± 20
- Selumetinib 25 mg/kg bid: 240 ± 20
- Cediranib 3 mg/kg/day: 220 ± 20
- Combination: 280 ± 20
Figure 5B

<table>
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<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>Paclitaxel 12.5 mg/kg/BID</th>
<th>Selumetinib 25 mg/kg/BID</th>
<th>Cediranib 3 mg/kg/day</th>
<th>Selumetinib (25 mg/kg/BID) and cediranib</th>
</tr>
</thead>
</table>

- NCI-H441
  - 100 μm

- NCI-H460
  - 100 μm

**Graphs:**

- **VEGFR2 H score:**
  - Vehicle
  - Paclitaxel
  - Selumetinib 12.5 mg/kg bid
  - Cediranib 3 mg/kg/day
  - Combination

- **VEGFR2 H score:**
  - Vehicle
  - Paclitaxel
  - Selumetinib 25 mg/kg bid
  - Cediranib 3 mg/kg/day
  - Combination
Figure 6

Vehicle  | Paclitaxel | Selumetinib 12.5 mg/kg/BID | Selumetinib 25 mg/kg/BID | Cediranib 3 mg/kg/day | Selumetinib (25 mg/kg/BID) and cediranib

NCI-H441

NCI-H460

50 μm
Clinical Cancer Research

Combined MEK and VEGFR Inhibition in orthotopic human lung cancer models results in enhanced inhibition of tumor angiogenesis, growth, and metastasis

Osamu Takahashi, Ritsuko Komaki, Paul D Smith, et al.

Clin Cancer Res Published OnlineFirst January 24, 2012.

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