Vascular Endothelial Growth Factor Receptor 2–Targeted Chemoprevention of Murine Lung Tumors

Vijaya Karoor\textsuperscript{1,4}, Mysan Le\textsuperscript{3}, Daniel Merrick\textsuperscript{2,3,4}, Edward C. Dempsey\textsuperscript{1,3,4}, and York E. Miller\textsuperscript{1,3,4}

Abstract

No clinically effective chemoprevention for lung cancer has been found. Angiogenesis is an early feature of both adenocarcinoma and squamous cell lung cancer. We investigated the effects of vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2) inhibition on lung carcinogenesis in a murine model of adenocarcinoma. The VEGFR-2 tyrosine kinase inhibitor, vandetanib, was given to FVB/N mice in chow for 7 days at varying doses to show pharmacologic activity by inhibition of VEGF-mediated VEGFR-2 and ERK phosphorylation. Plasma levels corroborated adequate dosage. For chemoprevention experiments, mice were injected i.p. with 1 mg/g of urethane, a carcinogen found in tobacco smoke. Chow containing vandetanib, 75 mg/kg/d, or control chow was given to mice, starting 7 days after urethane administration. Sixteen weeks after urethane injection, mice were sacrificed, tumors enumerated and measured. Vandetanib resulted in reductions in tumor multiplicity (6.5 \pm 0.86 versus 1.0 \pm 0.30, P = 0.001) and average tumor volume (0.85 \pm 0.10 versus 0.15 \pm 0.09 mm\textsuperscript{3}, P = 0.001), but not incidence (71\% versus 100\%, P = ns), compared with control. As vandetanib has other activities besides VEGFR-2 tyrosine kinase inhibition, we gave the anti-VEGFR-2 monoclonal antibody, DC101, for weeks 11 to 15 of a urethane carcinogenesis protocol with an arrest in tumor volume increase, but no change in multiplicity or incidence. Further investigation of the chemopreventive effect of vandetanib and other VEGF signaling inhibitors is needed. Cancer Prev Res; 3(9); 1141–7. ©2010 AACR.

Introduction

Lung cancer is the leading cause of cancer death in the world\textsuperscript{(1)}. Tobacco smoking is the major cause of lung cancer, and smoking cessation is an effective means to decrease lung cancer risk\textsuperscript{(2)}. However, significant risk of lung cancer persists after smoking cessation, such that in the United States, lung cancer is now diagnosed in approximately equal numbers of current and ex-smokers\textsuperscript{(3)}. Chemoprevention of lung cancer has the potential to significantly reduce morbidity and mortality. Unfortunately, no effective chemoprevention for lung cancer in humans has been found.

Angiogenesis has long been recognized as necessary for tumor growth\textsuperscript{(4)}. After reaching a diameter of 1 to 2 mm, tumors are dependent on the recruitment of new vessels and remain in a dormant state until the “angiogenic switch” occurs and new vessels are recruited. The molecular mechanisms of the angiogenic switch have been partially defined and include activating ras mutations as well as inactivation of p53, PTEN, and Smad4\textsuperscript{(5)}. The hypoxia-inducible factors, HIF-1\textsuperscript{α} and HIF-2\textsuperscript{α}, induce the expression of a variety of angiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor, (ELR\textsuperscript{+}) CXC chemokines (interleukin-8, CXCL12, and others), platelet-derived growth factor, endothelins, angiopoietins, and others\textsuperscript{(6)}. Conventionally thought of as critical when a tumor reaches 1 to 2 mm in diameter, angiogenesis is not commonly considered a feature of premalignancy. However, in the central airways, a premalignant lesion in which capillaries invade the overlying dysplastic endobronchial epithelium has been described and termed angiogenic squamous dysplasia (Fig. 1; ref. 7). This lesion occurs primarily in current or ex-smokers with endobronchial dysplasia and contains elevated levels of mRNAs for both VEGF-A and VEGF receptor-2 (VEGFR-2; ref. 8). The elevated levels of VEGF-A occur at multiple sites in individuals with angiogenic squamous dysplasia, suggesting a field effect. Angiogenesis also occurs in the evolution of at least some peripheral adenocarcinomas of the lung, which are thought to progress from atypical alveolar hyperplasia to bronchioloalveolar carcinoma to papillary adenocarcinoma and solid adenocarcinoma (Fig. 2). In papillary adenocarcinoma, malignant epithelial cells grow on an underlying capillary scaffold. Mouse lung adenomas are histologically similar to the papillary stage of human adenocarcinoma, with more advanced lesions displaying solid features (Fig. 3).

Several natural substances under investigation for cancer chemoprevention including silibinin, resveratrol, and...
green tea extract have antiangiogenic properties (9–11). However, few published studies have examined the chemopreventive properties of targeted antiangiogenic agents. We hypothesized that inhibition of angiogenesis might be an effective chemoprevention strategy for lung cancer in a murine model that has features of bronchioloalveolar carcinoma and adenocarcinoma. Chemical and genetic murine models of bronchioloalveolar carcinoma and adenocarcinoma have been investigated for many years and have many histologic, mutational, and gene expression features in common with human adenocarcinoma (12, 13). Murine models of squamous cell carcinoma are less well developed and have not yet been widely used for preclinical testing of chemopreventive agents (14, 15).

Materials and Methods

Human tissues
Human lung tumor tissues were harvested under protocols approved by the Colorado Multiple Institutional Review Board.

Mouse maintenance
Female FVB/N mice were purchased (Harlan) at ages 6 to 8 weeks and maintained in conventional caging in a controlled environment (12 hour light-dark cycle, food, and water ad libitum) in the Denver Veterans Affairs Medical Center Animal Care Facility. All animal procedures were approved by the Denver Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Demonstration of pharmacologic activity of vandetanib
Mice were fed powdered AIN-76A chow to which varying concentrations of vandetanib (a kind gift from Astra Zeneca, Macclesfield, United Kingdom) was added (BioServ, Frenchtown, NJ). Dosage was determined by observation of chow consumption by mice and calculation to achieve final doses of vandetanib of 50, 100, and 150 mg/kg/d (body weight). After 7 days on this diet, mice were injected i.p. with 20 ng of VEGF (Sigma) dissolved in PBS, then sacrificed 20 minutes later by i.p. injection of a mixture of pentobarbital (10 mg) and heparin (100 unit)/mouse in a 200 μL volume of saline. The aorta and inferior vena cava were then identified and transected, blood aspirated from the abdominal cavity and lungs were removed, dissected from the major bronchi, and

Fig. 1. Angiogenic squamous dysplasia in a human endobronchial biopsy. Note the capillary loops closely associated with the dysplastic squamous epithelium (arrows).

Fig. 2. Stages of human lung adenocarcinoma progression. A, atypical alveolar hyperplasia. B, bronchioloalveolar carcinoma. C, papillary adenocarcinoma. D, solid adenocarcinoma. The last three images were taken from different areas of the same tumor of a single patient. Note that the neoplastic cells in bronchioloalveolar and papillary carcinomas are arrayed on the surface of cores of mesenchymal cells containing central capillaries. It is apparent that in papillary adenocarcinoma, these structures have proliferated and fill the alveolar spaces.
Targeted Chemoprevention of Lung Tumors

Urethane carcinogenesis and chemoprevention

Mice were injected i.p. with 1 mg/g of urethane dissolved in saline. Serial body weights were followed during the experiment. One week after urethane injection, mice were placed on vandetanib or control AIN-76A chow for 10 weeks of a 20-week carcinogenesis experiment. Mice on control chow were sacrificed at 10- or 20-week time points.

Chemoprevention using anti-VEGFR-2 monoclonal antibody

The IgG1 rat anti-mouse VEGFR-2 monoclonal antibody DC101 was obtained from BioXcell. Eleven weeks after i.p. urethane (1 mg/g) injection, mice were given 700 μg/mouse DC101 or monoclonal rat IgG1 in 200 μL PBS, or PBS alone, i.p. thrice weekly for 4 weeks, then sacrificed, tumors enumerated and measured.

Immunohistochemistry

Tumors and lungs were fixed in 10% buffered formalin overnight, embedded in paraffin, and cut in serial 4-μm sections which were mounted on glass slides. All sections were pretreated with 10 mmol/L of Tris-EDTA (pH 9.0) for 20 minutes at 95°C. Sections were incubated with primary antibodies overnight at 4°C in a humidified chamber on a shaker. The sections were subsequently washed thrice in TBS and incubated with secondary antibodies, either biotinylated anti-rat IgG or anti-rabbit IgG (both at 1:200 dilution; Vector Laboratories) for 1 hour. Tissues were then washed thrice in TBS and incubated with horseradish peroxidase-conjugated streptavidin (Vector Laboratories) for 20 minutes at 95°C. Sections were incubated with primary antibodies overnight at 4°C in a humidified chamber on a shaker. The sections were subsequently washed thrice in TBS and incubated with secondary antibodies, either biotinylated anti-rat IgG or anti-rabbit IgG (both at 1:200 dilution; Vector Laboratories) for 1 hour. Tissues were then washed thrice in water, counterstained with modified Harris’ hematoxylin (Fisher Diagnostics) for 30 minutes. Tissues were washed thrice in TBS and developed in liquid 3,3′-diaminobenzidine (Biogenix) for 5 to 10 minutes until a brown color appeared. Tissues were then washed thrice in water, counterstained with modified Harris’ hematoxylin (Fisher Diagnostics) for 30 minutes, then washed thrice in water, dipped in acid ammonia, washed thrice in water, dehydrated in serial ethanol baths, cleared in CitriSolv (Fisher Diagnostics), and mounted on slides. Primary antibodies included 1:50 rat anti-mouse Ki-67 (Dako A/S), 1:100 rat anti-mouse CD34 (Santa Cruz Biotechnology), 1:100 rabbit anti-mouse pEGFR (Y1086), and 1:100 rabbit anti-mouse pVEGFR-2 (Cell Signaling). Controls included no primary antibody or same-species IgG.

Statistical tests

Data were analyzed using two-sided, nonparametric t tests.
Results

Dose determination
Mice tolerated vandetanib well and gained weight as did those fed control chow. Injection of 20 ng of VEGF i.p. resulted in increased phosphorylation of VEGFR-2 and Erk in lungs of controls. Following 7 days of treatment, vandetanib at a dose of 100 or 150 mg/kg/d was effective in blocking VEGF-induced VEGFR-2 phosphorylation. Vandetanib at 50, 100, and 150 mg/kg/d blocked Erk phosphorylation in response to VEGF injection. After 7 days of treatment, plasma levels of vandetanib at doses of 50, 100, and 150 mg/kg/d were 2.1, 4.5, and 5.7 μmol/L, respectively. For the remainder of the study, vandetanib was given in solid chow at a dose of 75 mg/kg/d.

Chemoprevention by vandetanib
In a 16-week carcinogenesis protocol, vandetanib chow (n = 7) had a major chemopreventive effect compared with control chow (n = 9). Mean tumor multiplicity and volume were reduced from 6.5 to 1.0 tumors/mouse (P = 0.0001) and 0.85 ± 0.10 versus 0.15 ± 0.09 mm³ (P = 0.001), respectively (Fig. 4). Tumor incidence was 100% with control chow and 71% with vandetanib (P = ns).

Vandetanib was effective administered both early and late
To compare early and late chemoprevention with vandetanib, we exposed mice to vandetanib-containing chow for either the first (n = 8) or last (n = 9) 10 weeks of a 20-week urethane carcinogenesis protocol. Mice fed control chow were sacrificed either at 10 (n = 8) or 20 (n = 10) weeks after urethane administration. Vandetanib given for either the first or last half of the carcinogenesis protocol was effective in reducing tumor multiplicity and volume in comparison with mice after 20 weeks of urethane carcinogenesis protocol (Fig. 5). Interestingly, vandetanib given for either the first or last 10 weeks of the 20-week carcinogenesis protocol reduced tumor multiplicities below that of mice fed control chow and sacrificed at 10 weeks after urethane, suggesting a therapeutic effect that persisted after vandetanib treatment was stopped.

Antibody inhibition of VEGFR-2 for chemoprevention
Vandetanib is not a specific inhibitor of VEGFR-2 tyrosine kinase inhibition. It also has significant inhibitory effects against other receptor tyrosine kinases including EGFR, VEGFR-3, and RET. The DC101 rat monoclonal antibody is a blocking antibody specific to the murine VEGFR-2 (17). Administration of DC101 is limited by the development of mouse anti-rat antibodies, so we administered DC101 between weeks 11 and 15 after urethane injection, a time period when the lung tumors began to grow rapidly. Mice tolerated DC101 or an isotype control IgG1 rat monoclonal well, with no differences in body weights during antibody or PBS administration. DC101 was effective in reducing tumor volume, but not multiplicity or incidence compared with PBS injection, in this modified protocol (Fig. 6). DC101 did prevent...
any tumor volume increase compared with controls sacrificed at 11 weeks.

**Effects of treatment on tumors developing during chemoprevention**

Due to the significant limitation of tissue availability for immunoblotting resulting from the reduction in tumor number and volume by vandetanib treatment, we chose to examine the characteristics of tumors that occurred in spite of chemoprevention by vandetanib (weeks 1-16) or DC101 (weeks 11-15) using immunohistochemistry. Histologically, tumors occurring in control, vandetanib, and DC101-treated mice appeared similar. Capillaries within the tumor were reduced by both vandetanib and DC101 treatment (Fig. 7). Immunostaining for phosphorylated EGFR and VEGFR was reduced to below that of controls following treatment with vandetanib but not with DC101 (Fig. 7). Expression of both total EGFR and VEGFR did not seem to be markedly altered by treatment with either vandetanib or DC101 (data not shown). The Ki-67 index was reduced by $\sim 50\%$ in both the vandetanib-treated ($P = \text{ns}$) and DC101-treated ($P = 0.003$) mouse tumors compared with controls; in the case of vandetanib-treated tumors, the number of tumors evaluable was limited by the lack of tissue for Ki-67 analysis.

**Discussion**

We believe that this is the first report to determine the effect of a targeted angiogenesis inhibitor for lung cancer chemoprevention. Vandetanib has previously been shown to be effective in reducing intestinal adenomas in the Apc-null mouse model (18). Vandetanib, given either continuously or intermittently, was highly effective in reducing lung tumor multiplicity and volume, but did not have a statistically significant effect on tumor incidence. This reduction in multiplicity and volume is significantly greater than our group has previously observed with SPC promoter-driven prostacyclin synthase overexpression, iloprost chow, or gefitinib injection (19-21). The efficacy of vandetanib given either early or late in a 20-week urethane carcinogenesis model shows that it does not have to be given continuously for chemopreventive efficacy. It is possible that further limited dosing strategies might limit toxicities, which include rash, diarrhea, and QT prolongation in human studies while maintaining chemopreventive efficacy.

Vandetanib is not completely specific for VEGFR-2 and inhibits other receptor tyrosine kinases, including the EGFR TK, VEGFR-3 TK, RET, and platelet-derived growth factor TK. We used a second agent, the anti-mouse VEGFR-2 monoclonal DC101 and showed significant effects on tumor volume when given for weeks 11 to 15 of a 15-week urethane carcinogenesis protocol, but no effect on tumor multiplicity, compared with control IgG1 or PBS injection from weeks 11 to 15. Moreover, DC101 administration prevented tumor volume increase compared with control mice sacrificed at 11 weeks. It is possible that earlier treatment with DC101, at a time when tumors were of a small enough volume as to be undetectable, might have reduced multiplicity as well. The development of mouse anti-rat antibodies limits the administration of DC101 to 4 weeks or less, making it difficult to directly compare the antibody and vandetanib experiments. Our previous results with the EGFR tyrosine kinase inhibitor gefitinib in a similar urethane model showed no chemopreventive activity in wild-type FVB/N mice and only a possible minor additive effect in prostacyclin synthase overexpressor mice (Lung Cancer, In press). Therefore, we do not believe that the chemopreventive effects of vandetanib are solely due to EGFR inhibition, although this could be a contributing factor. Furthermore, of the four other groups who have previously published chemoprevention experiments with EGFR tyrosine kinase inhibitor, only one has reported a large effect in a ras-driven model, such as that produced by urethane, a carcinogen found in tobacco smoke (22-25). Further investigation into the mechanisms by which vandetanib produces chemoprevention will require more specific, molecular, or genetic strategies which are beyond the scope of this report.
Activating EGFR mutations have been described as occurring frequently in histologically normal respiratory epithelium adjacent to human lung adenocarcinomas containing the same mutation (26). This supports EGFR mutation as an early premalignant event in some adenocarcinomas. A transgenic mouse model based on SPC promoter–driven mutant EGFR was amenable to highly effective chemoprevention by gefitinib (25, 27). However, most groups studying the chemopreventive effect of gefitinib in K-ras–driven models have found a small or no therapeutic effect (23, 24). In contrast, vandetanib is highly effective in the K-ras–driven urethane model. The dual VEGFR-2/EGFR inhibitory effects of vandetanib might make it, or a similar agent, effective in the chemoprevention of lung tumors driven by either mutant EGFR or K-ras.

Experiments characterizing the effects of vandetanib or DC101 on tumors were limited to tumors that developed during treatment. Comparability with tumors in control animals is difficult due to differences in volume and the possibility of acquired resistance to the agents. Both vandetanib and DC101 seemed to reduce capillary density and...
Ki-67 index. Vandetanib inhibited the phosphorylation of both EGFR and VEGFR-2, whereas DC101 only affected the latter.

Chemoprevention in humans requires the identification of high-risk populations and the availability of effective agents with minimal side effects. Populations at high risk (up to 2% incidence/year) for lung cancer could be readily identified using simple clinical features, including previous history of a tobacco-induced cancer, tobacco exposure history, coexistent chronic obstructive lung disease, and family history (28–30). Emerging genetic polymorphisms and susceptibility biomarkers might further improve the ability to identify subjects at high risk. However, effective agents with minimal side effects have not been discovered. Targeted agents often have toxicities that decrease their tolerability for chemoprevention. However, creative study designs, such as evaluating the effect on either surrogate end points or second primary tumors when given in a neoadjuvant setting, might allow the clinical evaluation of targeted agents for chemoprevention.

Disclosure of Potential Conflicts of Interest

Y.E. Miller is a co-inventor of a patent for the use of prostacyclin analogues for the prevention of cancer. No other authors have potential conflicts of interest.

Grant Support

Department of Veterans Affairs Merit Review and NCI P50 CA87187 (Y.E. Miller), NHLBI RO1 HL078929, and PPG HL14985 (V. Karoor and E.C. Dempsey).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 01/14/2010; revised 03/26/2010; accepted 04/29/2010; published OnlineFirst 07/20/2010.

References


www.aacrjournals.org
Vascular Endothelial Growth Factor Receptor 2–Targeted Chemoprevention of Murine Lung Tumors

Vijaya Karoor, Mysan Le, Daniel Merrick, et al.


Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-10-0005

This article cites 30 articles, 13 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/3/9/1141.full#ref-list-1

This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerpreventionresearch.aacrjournals.org/content/3/9/1141.full#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link
http://cancerpreventionresearch.aacrjournals.org/content/3/9/1141.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.