Differential response of primary tumor versus lymphatic metastasis to VEGFR-2 and VEGFR-3 kinase inhibitors cediranib and vandetanib

Timothy P. Padera,1 Angera H. Kuo,1 Tohru Hoshida,1,2 Shan Liao,3 Jennifer Lobo,1 Kevin R. Kozak,1 Dai Fukumura,1 and Rakesh K. Jain1

1Edwin L. Steele Laboratory for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts and 2Department of Advanced Surgical Science and Technology, Tohoku University, School of Medicine, Sendai, Japan

Abstract

Blood vessels are required for a tumor to grow and functional lymphatic vessels are required for it to disseminate to lymph nodes. In an attempt to eradicate both the primary tumor and its lymphatic metastasis, we targeted both blood and lymphatic vessels using two different tyrosine kinase inhibitors (TKIs): cediranib and vandetanib, which block vascular endothelial growth factor receptor (VEGFR)-2 and -3 in enzymatic assays. We found that although both cediranib and vandetanib slowed the growth rate of primary tumors and reduced blood vessel density, neither agent was able to prevent lymphatic metastasis when given after tumor cells had seeded the lymph node. However, when given during tumor growth, cediranib reduced the diameters of the draining lymphatic vessels, the number of tumor cells arriving in the draining lymph node, and the incidence of lymphatic metastasis. On the other hand, vandetanib had minimal effect on any of these variables, suggesting that vandetanib did not effectively block VEGFR-3 on lymphatic endothelial cells in our animal model. Collectively, these data indicate that the response of lymphatic vessels to a TKI can determine the incidence of lymphatic metastasis, independent of the effect of the TKI on blood vessels. [Mol Cancer Ther 2008;7(8):2272–9]

Introduction

Dissection of the molecular, cellular, and physical mechanisms of lymphatic metastasis is beginning to yield strategies to prevent lymphatic metastasis (1–7). Specifically, blocking vascular endothelial growth factor receptor (VEGFR)-3 signaling with monoclonal antibodies has been shown to prevent lymphatic metastasis but not control lymphatic metastasis after cancer cells have seeded the lymph node (8–10). To reveal which step of lymphatic metastasis is inhibited by blocking VEGFR-3 signaling, we recently monitored each step in the process of lymphatic metastasis using quantitative intravital microscopy (11) and found that blocking VEGFR-3 signaling reduces the delivery of cells to the lymph node.

These studies lead to a critical question: Can the blockade of both lymphangiogenic and angiogenic signaling pathways prevent formation of lymphatic metastasis after cancer cells have seeded the draining lymph node? This is a timely question in light of the vast pipeline of tyrosine kinase inhibitors (TKIs) of the VEGF receptor family currently undergoing clinical trials (12). In this first use of TKIs that block both VEGFR-2 and VEGFR-3 signaling to prevent and treat lymphatic metastasis, we used two orally available TKIs, cediranib and vandetanib (AstraZeneca; refs. 13, 14), in our ear model of lymphatic metastasis that permits imaging of each step in the metastatic process (11).

Materials and Methods

Cell Lines

VEGF-C–overexpressing (T241-VEGF-C-GFP) and mock-transduced (T241-GFP) T241 murine fibrosarcoma cell lines that constitutively express green fluorescent protein (GFP) under the EF1α promoter were established previously and cultured as reported in 10% fetal bovine serum/DMEM (11). Human dermal blood microvascular endothelial cells (BEC) and human dermal lymphatic microvascular endothelial cells (LEC) were purchased from Cambrex Corp. and grown in endothelial basal medium 2 containing EGM-2 MV SingleQuots. LECs and BECs were grown on 10 μg/mL fibronectin-coated plates.

Characterization of Gene Expression by Reverse Transcription-PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) and treated with DNase I before hybridization to the oligo(dT) primer. The first-strand cDNA was synthesized using SuperScript III First-Strand Synthesis System (Invitrogen). PCR was conducted with the following primer pairs using HotStar Taq Plus PCR kit (Qiagen). The following sense and antisense primers were used: VEGF-C, 5′-CAAGGCTTTTGAAGGCAAAG-3′ and

Received 2/25/08; revised 6/4/08; accepted 6/7/08.

Grant support: National Cancer Institute grants R01CA85140 (R.K. Jain) and P01CA80124 (R.K. Jain) and an unrestricted gift from AstraZeneca. 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 advertise payment of page charges. This article must therefore be hereby marked ¶-CAAGGCTTTTGAAGGCAAAG-3′ and

¶-CAAGGCTTTTGAAGGCAAAG-3′ and

Copyright © 2008 American Association for Cancer Research. doi:10.1158/1535-7163.MCT-08-0182
5'-TGGGTTCCTACGCTTCTCG-3'; VEGFR-1, 5'-TCTTCTCAAGTGCCAGAGG-3'; and 5'-CTAGTTGGTCCGATG-3'.

In vitro Proliferation Assays

The effect of cediranib and vandetanib on net proliferation of T241-GFP, T241-VEGF-C-GFP, BECs, and LECs was analyzed using a colorimetric WST-1–based assay (Cell Proliferation Reagent WST-1, Roche Applied Science). In short, cells were plated at 2,000/mL (T241-GFP or T241-VEGF-C-GFP) or 3,500/mL (BECs or LECs) and cultured in their growth medium as described above without any VEGF-C-GFP) or 3,500/mL (BECs or LECs) and cultured in their growth medium as described above without any added stimulation. After 24 h, different concentrations of cediranib or vandetanib were added. WST-1 assay was done following the manufacturer's protocol 72 h later.

TKI Dosing

Cediranib (3 mg/kg/d; ref. 14) and vandetanib (50 mg/kg/d; ref. 13) were given by oral gavage to animals in 1% Tween in PBS for 5 consecutive days followed by 2 consecutive days with no treatment. This schedule would constitute 1 wk of treatment. One percent Tween in PBS was used as the vehicle control for both compounds.

Animal Model

Experiments were done in nude mice and approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. To establish tumors, we injected 50 μL of tumor cell suspension (containing 5 x 10⁶ cells) in the tip of the ear. Tumor cell suspension was created from source tumors grown in the flank of four to six mice as previously described (11).

Treatment Protocols

Prevention Protocol. To test the ability of TKI administration to prevent lymph node metastasis, ear tumors were implanted and a TKI or vehicle was given during tumor growth. When tumors grew to 40 mm³ in volume, treatment was discontinued and the primary tumor was resected. Twenty-eight days after tumor removal (i.e., 42 d after original tumor implantation), microscopic and macroscopic metastases were assessed.

Intervention Protocol. To test the ability of TKI administration to control metastases after tumor cell seeding, ear tumors were implanted and left untreated for 14 d after implantation. On day 14, animals were distributed to ensure equal mean tumor size, primary tumors were resected, and a TKI or vehicle was given for the next 4 wk. Twenty-eight days after tumor removal (i.e., 42 d after original tumor implantation), microscopic and macroscopic metastases were assessed.

Lymphangiography and Tumor Cell Delivery to the Lymph Node

To examine the effects of TKI administration on tumor cell delivery, ear tumors were implanted and a TKI or vehicle was given during tumor growth. When the tumors grew to 40 mm³ in volume, functional lymphatic diameter and tumor cell delivery to the draining lymph node were measured as described below.

Lymphangiography was done by slow injection in the interstitial tissue of the peripheral ear of 500,000-Da tetramethylrhodamine isothiocyanate-dextran (Invitrogen). Ear lymphatics were observed with epifluorescence intravital microscopy and/or multiphoton intravital microscopy (15, 16). Lymphangiography images were captured and lymphatic diameters were measured using ImageJ software as previously described (11).

To quantify tumor cell delivery following lymphangiography, the cervical lymph node was exposed and imaged with multiphoton intravital microscopy. Images of all the GFP-positive cells detectable in each lymph node were captured as image stacks with a 10-μm interval step. One to eight image stacks per lymph node and 10 to 51 slices per field were acquired. The number of cells was counted using ImageJ software in a blinded fashion by two investigators.

Histologic Analysis

For the metastasis assay, formalin-fixed and paraffin-embedded cervical lymph nodes were stained with H&E. Multiple sections spaced 200 μm apart spanning the entire lymph node were examined. For blood vessel evaluation in primary tumors, size-matched tumors were fixed in 4% paraformaldehyde by immersion, embedded in paraffin, and immunostained with MECA-32 antibody (1:50; BD Biosciences), VEGFR-2 (1:500; Cell Signaling), VEGFR-3 (1:100; ebioscience), and PDGFR-β (1:50; Cell Signaling) staining were carried out on T241-VEGF-C-GFP ear tumors and developed with diaminobenzidine.

Immunofluorescence

T241-VEGF-C-GFP tumors were fixed in 4% formaldehyde in PBS for 2 h and followed by 30% sucrose overnight. Tissues were embedded in OCT, frozen, and cut into 20-μm-thick sections. Sections were fixed in cold acetone and blocked with 3% bovine serum albumin + 5% normal horse serum + 0.1% Triton X-100 in PBS. Primary antibody was incubated overnight followed by secondary antibody incubation for 2 h. The primary antibodies were as follows: rat anti-CD31 (BD Biosciences), hamster anti-CD31 (Chemicon), rabbit anti-LYVE-1 (Upstate Biotechnology), 3

<http://rsb.info.nih.gov/ij/>
rabbit anti-VEGFR-2 (Cell Signaling), and rat anti-VEGFR-3 (eBioscience). Species-appropriate secondary antibodies were labeled with Cy3 or Cy5 (Jackson ImmunoResearch).

Statistics
Quantitative data are presented as the mean ± SE. Paired and unpaired Student’s t test, Mann-Whitney U test, ANOVA with Games-Howell post hoc test, and Fisher’s exact test were used for statistical analysis. Values of P ≤ 0.05 were considered statistically significant.

Results and Discussion

In vitro and In vivo Validation of TKI Targets on T241 Tumor Cell Lines
We hypothesized that blocking both VEGFR-2 and VEGFR-3 can inhibit lymphatic metastasis by acting on both blood and lymphatic vessels. To test this hypothesis, we used two TKIs with similar relative VEGFR-2/VEGFR-3 isolated enzymatic IC50s: cediranib and vandetanib (13, 14). Cediranib has additional enzymatic activity (IC50 within 10- to 100-fold of VEGFR-2) against VEGFR-1, c-Kit, and PDGFR-β (14). In contrast, vandetanib has additional enzymatic activity against epidermal growth factor receptor (13) and Ret (17). In light of these IC50 data, reverse transcription-PCR was used to characterize the in vitro expression of the multiple targets of cediranib and vandetanib in T241-GFP and T241-VEGFR-C-GFP cell lines. Of these targets, including VEGFR-2 and VEGFR-3, only PDGFR-α and PDGFR-β were detectable in these cell lines (Fig. 1A). We then immunohistochemically stained for VEGFR-2, VEGFR-3, and PDGFR-β in T241-VEGFR-C-GFP tumors and found that these receptors were not detectable on the cancer cells in vivo (Fig. 1B). We did find that VEGFR-2 was present on 89% of CD31-positive vessels, whereas VEGFR-3 was only present on 15% of CD31-positive vessels based on immunofluorescence (Supplementary Fig. S1).4 Furthermore, we found that 93% of VEGFR-3–positive vessels were also LYVE-1 positive. Although PDGFR-β was not present on tumor cells, it was detected on less than one vessel per high-power field (0.35 mm² field size).

Effects of Cediranib and Vandetanib on Endothelial Cell and Tumor Cell Proliferation
We then tested the effect of cediranib and vandetanib on proliferation of BECs, LECs, T241-GFP cells, and

4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
T241-VEGF-C-GFP cells *in vitro* (Fig. 2). The LEC and BEC proliferation IC$_{50}$s for vandetanib were ~5-fold higher than those for cediranib, in concert with published data (13, 14). The T241-GFP and T241-VEGF-C-GFP cell proliferation IC$_{50}$s for cediranib were at least 5-fold higher than for BECs and LECs (Fig. 2). These data suggest that the effect of cediranib on the rate of tumor growth and metastasis is likely due to its effect on the vasculature and not the tumor cells despite the presence of PDGFR-α and PDGFR-β on the tumor cells. In contrast, the tumor cell proliferation IC$_{50}$s for vandetanib were comparable with BECs and LECs (Fig. 2B). The T241-GFP and T241-VEGF-C-GFP IC$_{50}$s for vandetanib were comparable with that for cediranib.

The comparable response of the T241 tumor cell lines and endothelial cells to vandetanib is somewhat surprising as the tumor cells lack the primary targets of vandetanib. This result is consistent with published literature of IC$_{50}$s for vandetanib on unstimulated human umbilical vascular endothelial cells (>3 μmol/L) and six tumor cell lines (2.7–13.5 μmol/L), showing that unstimulated endothelial cells have similar or higher IC$_{50}$s compared with tumor

**Figure 2.** Cediranib reduces the proliferation of BECs and LECs more potently than T241 tumor cell lines. A, dose-response curves of cediranib and vandetanib on LECs and BECs. B, dose-response curves of cediranib and vandetanib on T241-GFP and T241-VEGF-C-GFP tumor cells. Proliferation was determined by WST-1 assay.
Both Cediranib and Vandetanib Treatments Cause Tumor Growth Delay

We next implanted T241-VEGF-C-GFP tumor cells in our ear model of lymphatic metastasis (11) and measured the effect of cediranib and vandetanib on the time required for these tumors to grow to 40 mm³. In both control groups, tumor growth took ~2 weeks (P > 0.05). As expected, both cediranib and vandetanib caused a significant delay in primary tumor growth (Table 1A; Supplementary Fig. S2). 4 However, at the doses used, the growth delay caused by vandetanib (~2 weeks) was nearly twice as long as that caused by cediranib (~1 week; P < 0.05). Using MECA-32 immunohistochemistry, we found a reduction in the area density of intratumor blood vessels in both cediranib- and vandetanib-treated tumors when compared with controls (Fig. 3), suggesting that the growth delay may be due to an antiangiogenic effect of the TKIs. The equivalent reduction in vascular density suggests that the greater growth delay created by vandetanib may also be due in part to direct tumor cell effect, as implied by our cell proliferation data (Fig. 2), or through inhibition of epidermal growth factor receptor and/or Ret on stromal cells.

Cediranib Prevents Formation of Lymphatic Metastasis

The ability of both TKIs to inhibit primary tumor growth suggested that these compounds may be able to inhibit both the formation of lymphatic metastasis and its subsequent growth in the lymph node. To test this hypothesis, we used two different treatment protocols: intervention and prevention. In the intervention protocol, which simulates the situation in which a patient is diagnosed with a primary tumor that may or may not have already spread, both cediranib and vandetanib were unable to reduce lymph node metastasis from T241-VEGF-C-GFP tumors (Table 1B). These data are consistent with our previous report using a blocking monoclonal antibody to VEGFR-3 (11).

In the prevention protocol, in which the TKIs were given from the time of tumor implantation until the tumors reached a size of 40 mm³, cediranib was able to reduce the incidence of lymphatic metastasis from T241-VEGF-C-GFP tumors (Table 1B). In contrast, vandetanib was not able to prevent metastasis.

In spite of the ability of both TKIs to inhibit tumor growth, we found a marked difference in the ability of the two compounds to prevent the formation of lymphatic metastasis. This result was surprising, particularly in light of the longer growth delay induced by vandetanib. Vandetanib dose escalation was not possible as the animals were losing weight over the 30 days of treatment at a dose of 50 mg/kg (5 days per week). The group treated with vandetanib lost 7% of its body weight (P < 0.001) compared with the control group, which showed no weight change (P > 0.1). Animals treated with cediranib at a dose of 3 mg/kg (5 days per week) did not show any weight change (P > 0.1).

Cediranib Inhibits Lymphatic Hyperplasia in the Tumor Margin and Arrival of Tumor Cells in the Draining Lymph Node

The difference in ability of cediranib and vandetanib to prevent lymphatic metastasis might be attributable to their differential effects on a specific step in the process of lymphatic metastasis. Using our previously described ear model and intravital microscopy (11), we tested the effect of both TKIs on peritumor lymphatic vessel size and tumor cell arrival in the lymph node. As both cediranib and vandetanib reduced primary tumor growth rate, size-matched tumors (40 mm³) were used to compare treated tumors with controls. This mandated that treated tumors grew for a longer period before evaluation. Because a major correlate to the arrival of cells in the lymph node is time of primary tumor growth (11), using size-matched tumors biases the experiments against the hypothesis that these TKIs can reduce tumor cell arrival and thus provides a rigorous test.

Cediranib reduced the diameter of peritumor lymphatic vessels in size-matched T241-VEGF-C-GFP tumors (Fig. 4A). This was accompanied by a decrease in the number of cells arriving in the draining lymph node (Fig. 4B) quantified by multiphoton intravital microscopy. Cediranib treatment of T241-VEGF-C tumors, which produce minimal levels of VEGF-C, showed no reduction in peritumor lymphatic diameter or lymph node cell arrival (data not shown). This result is likely due to the relatively normal peritumor lymphatic vessels and low rate of tumor

### Table 1. Cediranib and vandetanib effects on tumor growth and metastasis

<p>| A. Cediranib and vandetanib treatments result in tumor growth delay in T241-VEGF-C-GFP tumors |
| Time for tumor to reach 40 mm³ (d) | P |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cediranib</td>
<td>23 ± 1.5 (n = 13)</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>30 ± 1.6 (n = 11)</td>
</tr>
</tbody>
</table>

<p>| B. Cediranib, but not vandetanib, prevents the generation of lymphatic metastasis, but neither can stop metastasis after cancer cells have spread from T241-VEGF-C-GFP tumors |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intervention protocol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cediranib</td>
<td>7/15</td>
<td>7/15</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>7/13</td>
<td>6/14</td>
</tr>
<tr>
<td>Prevention protocol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cediranib</td>
<td>10/19</td>
<td>15/17</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>11/11</td>
<td>11/14</td>
</tr>
</tbody>
</table>

NOTE: Data presented are the number of animals with lymphatic metastasis per the total number in its experimental group.
cell arrival associated with T241-GFP (11). These data show that cediranib can inhibit lymphatic vessel hyperplasia and regional lymph node seeding induced by VEGF-C overexpression but has little effect on quiescent or normal lymphatic vessels. Thus, cediranib can be used to target lymphangiogenic vessels stimulated by VEGF-C. Collectively, cediranib elicits an antilymphatic hyperplasia response (Fig. 4A) in addition to its antiangiogenic effects (Fig. 3A). This combination is attractive to prevent lymphatic metastasis.
Vandetanib did not reduce lymphatic hyperplasia and tumor cell arrival from T241-VEGF-C-GFP tumors (Fig. 5). These data explain the lack of reduction in lymphatic metastasis using vandetanib in the prevention protocol. Because there was no antilymphatic hyperplasia effect, there was no reduction in tumor cell arrival in the draining lymph node and thus no reduction in lymphatic metastasis in our model.

Many potential explanations exist for the differential effect of the two TKIs on lymphatic hyperplasia in our model. Although both TKIs caused reductions in tumor growth and blood vessel density (Fig. 3), the pharmacokinetics of targeting blood vessels differs from that of lymphatic vessels. To cause a lymphatic effect, a drug has to be transported across the blood vessel wall and through the intervening tissue to the lymphatic vessels in sufficient quantity. Vandetanib may be transported through the tissue less efficiently than cediranib, resulting in the lack of a lymphatic effect. As the animals receiving vandetanib at 50 mg/kg/d five times a week were losing body mass, a higher dose could not be given to overcome transport barriers. Thus, the transvascular and interstitial transport properties of cediranib and vandetanib may account for the differential efficacy of the two drugs.

Our previous findings in the same tumor model show a reduction in peritumor lymphatic hyperplasia, a reduction in cell arrival, and a reduction in lymph node metastasis when only VEGFR-3 is blocked using a monoclonal antibody against VEGFR-3 (11). If vandetanib was effectively blocking VEGFR-3 signaling in LECs in our model, we would expect a similar result. Thus, the data presented here suggest that vandetanib did not effectively block VEGFR-3 signaling in tumor-associated lymphatic vessels in our model. This may be a result of insufficient concentrations of vandetanib in lymphatic vessels or limited effectiveness of vandetanib in blocking VEGFR-3 signaling in vivo.

Another possibility is that subtle differences in the pharmacodynamic effects of the two TKIs, particularly against targets with higher in vitro IC$_{50}$s than VEGFR-2 and VEGFR-3 (13, 14), may account for the differential response in lymphatic vessel hyperplasia and lymphatic metastasis. For instance, VEGFR-1 signaling is important in pathologic angiogenesis (18) and macrophage activation (19). Macrophages have been implicated as playing an important role in lymphangiogenesis (20–23). We found no significant difference in the density of macrophages in tumor tissue after treatment with cediranib (data not shown), arguing against this possibility. PDGFR-β is involved in tumor lymphangiogenesis in animal models and is present on lymphatic vessels (24). Using imatinib mesylate, a TKI that inhibits PDGFR-β, bcr-abl, c-Kit, and stem cell factor, we observed no reduction in lymphatic metastasis when treatment was given during tumor growth (data not shown). Other possible targets for inhibition include epidermal growth factor receptor or Ret signaling in the stromal cells of T241-VEGF-C-GFP tumors, which may be important for tumor growth. Vandetanib activity against these molecules could account for the greater primary tumor growth inhibition when compared with cediranib without affecting lymphatic morphology or metastasis.

Our data show that cediranib, which blocks signaling from VEGFR-2 and VEGFR-3, can inhibit lymphatic hyperplasia and lymphatic metastasis induced by VEGF-C--overexpressing T241 fibrosarcomas. This result is consistent with our previous report that used a blocking VEGF-3 monoclonal antibody under the same conditions (11). Cediranib also had the benefit of inhibiting primary
tumor growth through an antiangiogenic mechanism. Thus, the TKI cediranib seems to be a compelling compound for use in antitumor and antilymphatic metastatic strategies.

**Disclosure of Potential Conflicts of Interest**

R.K. Jain: grant support from AstraZeneca, consultant to AstraZeneca and Dyax, and Science Advisory Board member of SynDevRx. The other authors disclosed no potential conflicts of interest.

**Acknowledgments**

We thank Emmanuelle di Tomaso, Johanna Lahdenranta, James Tyrrell, Juliane M. Jürgensmeier, and Anderson Ryan for their scientific and technical input and Sylvie Roberge and Carolyn Smith for their outstanding technical support.

**References**

Molecular Cancer Therapeutics

Differential response of primary tumor versus lymphatic metastasis to VEGFR-2 and VEGFR-3 kinase inhibitors cediranib and vandetanib

Timothy P. Padera, Angera H. Kuo, Tohru Hoshida, et al.


Updated version

Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-08-0182

Supplementary Material

Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2008/08/20/1535-7163.MCT-08-0182.DC1

Cited articles

This article cites 24 articles, 11 of which you can access for free at:
http://mct.aacrjournals.org/content/7/8/2272.full.html#ref-list-1

Citing articles

This article has been cited by 22 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/7/8/2272.full.html#related-urls

E-mail alerts

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

Reprints and Subscriptions

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

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.