Multi-Kinase Inhibitor E7080 Suppresses Lymph Node and Lung Metastases of Human Mammary Breast Tumor MDA-MB-231 via Inhibition of Vascular Endothelial Growth Factor-Receptor (VEGF-R) 2 and VEGF-R3 Kinase

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Abstract

Purpose: Vascular endothelial growth factor (VEGF)-C/VEGF-receptor 3 (VEGF-R3) signal plays a significant role in lymphangiogenesis and tumor metastasis based on its effects on lymphatic vessels. However, little is known about the effect of inhibiting VEGF-R3 on lymphangiogenesis and lymph node metastases using a small-molecule kinase inhibitor.

Experimental Design: We evaluated the effect of E7080, a potent inhibitor of both VEGF-R2 and VEGF-R3 kinase, and bevacizumab on lymphangiogenesis and angiogenesis in a mammary fat pad xenograft model of human breast cancer using MDA-MB-231 cells that express excessive amounts of VEGF-C. Lymphangiogenesis was determined by lymphatic vessel density (LVD) and angiogenesis by microvessel density (MVD).

Results: In contrast to MDA-MB-435 cells, which expressed a similar amount of VEGF to MDA-MB-231 cells with an undetectable amount of VEGF-C, only MDA-MB-231 exhibited lymphangiogenesis in the primary tumor. E7080 but not bevacizumab significantly decreased LVD within the MDA-MB-231 tumor. E7080 and bevacizumab decreased MVD in both the MDA-MB-231 and MDA-MB-435 models. E7080 significantly suppressed regional lymph nodes and distant lung metastases of MDA-MB-231, whereas bevacizumab significantly inhibited only lung metastases. E7080 also decreased both MVD and LVD within the metastatic nodules at lymph nodes after resection of the primary tumor.

Conclusions: Inhibition of VEGF-R3 kinase with E7080 effectively decreased LVD within MDA-MB-231 tumors, which express VEGF-C. Simultaneous inhibition of both VEGF-R2 and VEGF-R3 kinases by E7080 may be a promising new strategy to control regional lymph node and distant lung metastases.

Invasion and metastases to regional lymph nodes and distant organs are common features of malignant tumors. Tumor metastases to distant organs are the main cause of death in cancer patients. The extent of lymph node metastases is one of the most important prognostic factors and often determines the choice of therapy for cancer patients (1, 2). Lymph node metastases occur through lymphangiogenesis (the formation of new lymphatic vessels; refs. 3, 4), and metastases to distant organs occur through angiogenesis (the formation of new blood vessel). Lymphangiogenesis and angiogenesis are regulated by five endothelial cell–specific growth factors [vascular endothelial growth factor-A (VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor] and three of their receptor tyrosine kinases (VEGF-R1, VEGF-R2, and VEGF-R3; refs. 5, 6). Because the VEGF/VEGF-R2 signal is critical for tumor angiogenesis, inhibition by either an antibody to VEGF or a small molecular inhibitor for VEGF-R2 kinase suppresses tumor growth and prolongs the life span of cancer patients in clinical therapy (7–9). VEGF-C and VEGF-D activate VEGF-R3 and are identified as lymphangiogenic factors, although fully processed forms of VEGF-C also activate VEGF-R2 (10). The association of VEGF-C expression with lymphangiogenesis and lymph node metastasis is observed in many cancers, such as breast (11), prostate (12), gastric (13), lung (14), and melanoma (15). In a preclinical study, overexpression of VEGF-C promoted intratumoral lymphangiogenesis and lymph node metastasis of breast and lung tumor cells (16). Soluble VEGF-R3–mediated inhibition of the VEGF-C/VEGF-R3 signal suppressed lymph node metastases of breast and lung tumors mainly due to blocking of tumor lymphangiogenesis. Anti-VEGF-R3 antibody also suppressed lymph node metastases driven by overexpressed VEGF-C in mice (4, 17). In mice, inhibition of metastasis to regional lymph nodes and distant organs, such as the lung, is more effectively blocked by a combination of

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antibodies to both VEGF-R2 and VEGF-R3 than by each antibody separately (18).

Several small molecular inhibitors of VEGF-R kinases, such as sunitinib (8) and sorafenib (9), are now available for use in clinical treatment. Although sunitinib (19), sorafenib (20), and CEP-7055 (21) have strong activity against VEGF-R3 kinase (IC_{50} with cell-free kinase assay: 30 nmol/L, 10-20 nmol/L, and 8 nmol/L, respectively), the extent of their efficacy against lymphangiogenesis has not been definitively evaluated (3). We found that E7080 was the most potent dual inhibitor of VEGF-R3 tyrosine kinase (IC_{50}, 5.2 nmol/L) as well as VEGF-R2 tyrosine kinase (IC_{50}, 4.0 nmol/L; ref. 22) among those small-molecule inhibitors (3). In this article, we determined the effect of E7080 on both angiogenesis and lymphangiogenesis using a MDA-MB-231 mammary fat pad (m.f.p.) model. This is the first report showing that a dual tyrosine kinase inhibitor of VEGF-R2 and VEGF-R3 is more effective than bevacizumab, a VEGF-R2 signaling inhibitor, in reducing lymphangiogenesis.

Materials and Methods

Reagents. E7080 was synthesized at Eisai Co. Ltd. (22). Bevacizumab (Avastin) was purchased from Genentech.

Cells. Human umbilical vascular endothelial cells (HUVEC) were isolated from human umbilical cords by a method described previously (23). Human breast adenocarcinoma, MDA-MB-231, was obtained from the American Type Culture Collection and human melanoma, MDA-MB-435, was obtained from Dr. Mary J.C. Hendrix (University of Arizona, Tucson, AZ). MDA-MB-231 was engineered to express red-shifted green fluorescence protein (rsGFP) for detection *in vivo* using an imaging system (AntiCancer, Inc.), and the population of MDA-MB-231 cells highly expressing rsGFP was sorted by flow cytometry.

Cell-free kinase assay/cell phosphorylated assay. Tyrosine kinase activity was measured by a homogeneous time-resolved fluorescence assay (VEGF-R2, VEGF-R1, fibroblast growth factor-receptor 1 (FGF-R1), and epidermal growth factor receptor) and by ELISA [platelet-derived growth factor (PDGF) receptor β] using the recombinant kinase domains of these receptors (22). The kinase inhibitory activity of E7080 against VEGF-R3 was examined using the technology platform from the ProQinase Co. (24). For cell-free kinase assay, samples were duplicated and two to three separate experiments were done. HUVECs were cultured with serum-free medium containing 0.5% fetal bovine serum for 24 h. Cells were treated with E7080, stimulated by either VEGF (20 ng/mL) or VEGF-C (100 ng/mL) for 10 min, and then collected in lysis buffer. To detect VEGF-R2 and phosphorylated VEGF-R2, 10 to 20 μg of cell lysates were electrophoresed. To detect VEGF-R3 and phosphorylated VEGF-R3, 400 to 1,000 μg of cell lysates were immunoprecipitated by anti-VEGF-R3. Immune complexes were solubilized in 60 μL of sample buffer and electrophoresed. The resolved proteins were analyzed by Western blot with the indicated antibodies: for VEGF-R2 (Santa Cruz Biotechnology) and phosphorylated VEGF-R2 (Cell Signaling) and for VEGF-R3 (Santa Cruz Biotechnology) and anti-phosphotyrosine IgG (4G10; Upstate Biotechnology). Immunoreactive bands were visualized by chemiluminescence (Pierce Biotechnology) using the Image Master VDS-CL (Amersham Pharmacia Biotech). The intensity of each band was measured using 1D Image Analysis software (version 1.6; Kodak). For cell phosphorylated assay, three separate experiments were done.

Proliferation assay stimulated with growth factors. HUVECs (1,000 cells in each well in serum-free medium containing 2% fetal bovine serum) and L6 rat skeletal muscle myoblasts (5,000 cells in each well in serum-free DMEM) were dispensed in a 96-well plate and incubated overnight. E7080 and either VEGF (20 ng/mL) or FGF-2 (20 ng/mL) containing 2% fetal bovine serum and PDGFβ (40 ng/mL) were added to each well. Cells were incubated for 3 d and then the ratios of surviving cells were measured by WST-1 reagent (Dojindo). For proliferation assay, samples were duplicated and three separate experiments were done.

Secretion of VEGF, VEGF-C, and VEGF-D in conditioned medium. MDA-MB-231 and MDA-MB-435 cells (3×10^5) were cultured in a six-well plate with RPMI 1640 containing 0.1% bovine serum albumin for 24 h. Conditioned media were collected and the amounts of growth factors were measured using ELISA detection kits for VEGF-A (Immuno-Biological Laboratories Co. Ltd.), VEGF-C (Immuno-Biological Laboratories), and VEGF-D (R&D Systems).

Immunohistochemical analysis of angiogenesis and lymphangiogenesis in m.f.p. xenograft models. MDA-MB-231 and MDA-MB-435 tumors were removed from mice treated with either E7080 ($n = 5$) or bevacizumab ($n = 5$) for 1 wk (day 8) and without treatment ($n = 5$), embedded in OCT compound, frozen on dry ice, and double stained for an endothelial cell marker CD31 (with rat monoclonal anti-mouse CD31, clone MEC13.3; BD PharMingen) and a lymph endothelial cell marker (with rabbit polyclonal anti-LYVE-1; Upstate Biotechnology). CD31 and LYVE-1 were visualized by staining with fuchsin (DAKO) and 3,3′-diaminobenzidine (Dojindo), respectively. Microvessel density (MVD) and lymphatic vessel density (LVD) were assessed by counting tumor microvessel and lymph vessel elements (four to five fields per tumor) and calculating tumor microvessel or lymph vessel densities (i.e., number of vessel elements per field). Experiments were duplicated and statistical analysis was done using the Dunnett-type multiple comparison method.

Effect of E7080 on the primary tumor growth in the m.f.p. and metastases. MDA-MB-231 cells highly expressing rsGFP were implanted s.c. into the flanks of nude mice. Tumor fragments (17 ± 2 mg) were prepared from 100 to 200 mm^3 tumors grown s.c. and then inoculated into the m.f.p. About 2 wk after inoculation, mice were randomized into control ($n = 12$) and treatment groups ($n = 10$) at day 1. Either E7080 (in water) or bevacizumab (in saline) was administered orally once a day or i.v. twice a week, respectively, from day 1 to day 56. Antitumor activity was shown as a relative tumor volume (RTV = calculated tumor volume/day 1 tumor volume). Tumors expressing rsGFP in the lymph node and lung were detected by a fluorescence imaging detection system (Hamamatsu Photonics K.K.) after 56 d of treatment. Data include the average with SD for RTV and the ratio of the number of mice bearing metastatic nodules. Experiments were duplicated and statistical analysis was conducted using the Dunnett-type multiple comparison method.

Effect of E7080 on tumor growth of metastatic nodules in the lymph nodes after resection of the primary tumor. rsGFP MDA-MB-231 tumor pieces were transplanted and allowed to grow until metastases were noted in the lymph nodes (~90 d), which were detected by a fluorescence imaging detection system, and then the primary tumors were removed. Eight mice were divided into two groups. Administration of E7080 was started 2 wk after resection of the primary tumors (day 1). E7080 was administered orally once a day from day 1 to day 28. Statistical analysis was conducted using the Dunnett-type multiple comparison method.

Results

Kinase inhibitory profile of E7080. The kinase inhibitory profile of E7080 was determined using a cell-free kinase assay (Table 1). E7080 potently inhibited VEGF-R3 kinase activity (IC_{50}, 5.2 nmol/L; Table 1; Supplementary Fig. S1) and VEGF-R2 kinase activity (IC_{50}, 4.0 nmol/L) to a similar extent (Table 1). E7080 also inhibited VEGF-R1, FGF-R1, and PDGF-Rβ kinase, but the inhibitory activity was about 4 to 10 times less potent (Table 1). EGFR kinase was not effectively inhibited with E7080. E7080 showed strong inhibition of phosphorylation of VEGF-R2 (IC_{50}, 0.83 nmol/L) and VEGF-R3 (IC_{50}, 0.36 nmol/L) in

Table 1. Summary of the inhibition of VEGF-R2 and VEGF-R3 kinases by E7080

Receptor kinase	IC_{50} (nmol/L)		
	Cell-free kinase	Cell-based assay	
		Phosphorylation	Proliferation
VEGF-R1	22	—	—
VEGF-R2	4.0	0.83	5.2
VEGF-R3	5.2	0.36	—
FGF-R1	46	—	410
PDGFRβ	39	—	340
EGFR	6,500	—	—

NOTE: IC_{50} values of E7080 were determined by either cell-free or cell-based assays. Cell-free kinase assay and cell-based assays were done as described in Materials and Methods. HUVECs were used in the cell-based assays except for the assay of PDGFRβ kinase, where L6 rat skeletal muscle myoblasts were used.
Abbreviation: EGFR, epidermal growth factor receptor.

HUVECs after stimulation with VEGF and VEGF-C, respectively (Table 1; Fig. 1). These data indicated that E7080 was a potent inhibitor of VEGF-R3 kinase as well as VEGF-R2 kinase. Inhibitory activity of E7080 against VEGF-induced proliferation of HUVEC (IC_{50}, 2.7 nmol/L) was stronger than basic FGF induced (IC_{50}, 410 nmol/L) in HUVEC and PDGF-induced proliferation of L cells (IC_{50}, 340 nmol/L; Table 1). We were not able to determine the IC_{50} value for VEGF-C–induced cell proliferation because VEGF-C did not stimulate cell proliferation in our assays.

E7080 inhibits both angiogenesis and lymphangiogenesis induced by human breast cancer cells. MDA-MB-231 cell is a human breast adenocarcinoma cell derived from pleural effusion (25). Metastases of MDA-MB-231 cells inoculated into the m.f.p. developed in the regional lymph nodes and

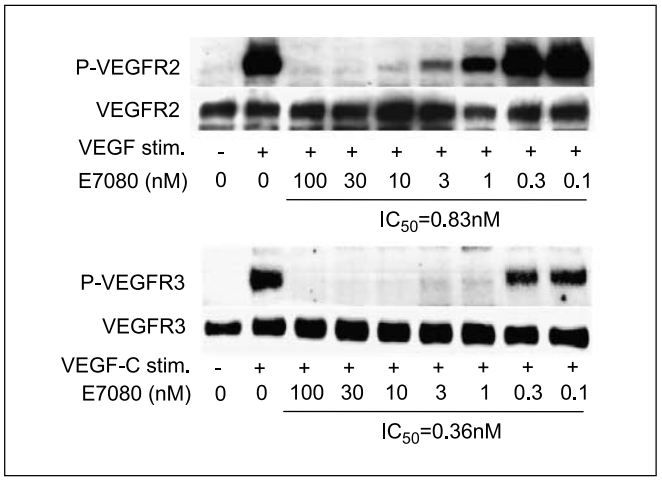

Fig. 1. Cell-based phosphorylation analysis of VEGF-R2 and VEGF-R3 kinases by E7080. Western blotting analysis of phosphorylation status of VEGF-R2 and VEGF-R3. Top, dose-dependent effect of E7080 on the phosphorylation status of VEGF-R2; bottom, dose-dependent effect of E7080 on the phosphorylation status of VEGF-R3. Total cell lysates were incubated at 4°C with anti-VEGF-R3 and immunoprecipitated with protein A/G-Sepharose beads. Western blotting analysis was done as described in Materials and Methods.

Table 2. Effect of E7080 and bevacizumab on metastasis to either regional lymph nodes or distant lung in the MDA-MB-231 xenograft model

Treatment	Incidence of metastasis	
	Lymph node metastasis	Lung metastasis
(—)	9/12	9/12
E7080	0/10*	0/10*
Bevacizumab	6/10	3/10*

NOTE: Metastases were evaluated as described in Materials and Methods 56 d after the start of treatment.
*$P < 0.05$.

distant lung with high frequency (Table 2), whereas those of MDA-MB-435 was developed only in the distant lung (data not shown). ELISA assay of conditioned medium indicated that both tumor cells expressed significant amounts of VEGF, but only MDA-MB-231 produced high amounts of VEGF-C (Table 3), and neither of cell lines produced detectable amounts of VEGF-D. These data suggested that the VEGF/VEGF-R2 and VEGF-C/VEGF-R3 signals might be activated, resulting in metastases to the regional lymph nodes and distant lung in the MDA-MB-231 m.f.p. xenograft model, whereas only the VEGF/VEGF-R2 signal might be activated, resulting in metastasis to the distant lung in the MDA-MB-435 m.f.p. xenograft model. To determine roles of VEGF/VEGF-R2 and VEGF-C/VEGF-R3 signals in metastasis, we examined the effects of an anti-VEGF antibody, bevacizumab (a selective inhibitor of the VEGF signal), and E7080 (a dual inhibitor of VEGF-R2 and VEGF-R3 kinases), on angiogenesis and lymphangiogenesis in two m.f.p. xenograft models. The extent of angiogenesis and lymphangiogenesis was evaluated by staining tumor tissues with anti-CD31 antibody and anti-LYVE-1 antibody, respectively.

Immunohistochemical analysis with anti-CD31 antibody showed that MDA-MB-231 and MDA-MB-435 had similar MVD (82.1 ± 11.3 and $61.4 \pm 7.4/mm^2$, respectively) on the first day of inhibitor administration (Fig. 2B). By contrast, immunohistochemical analysis with anti-LYVE-1 antibody indicated that the number of lymphatic vessels was significantly lower in MDA-MB-435 ($2.5 \pm 3.4/mm^2$) than in MDA-MB-231 ($36.0 \pm 14.0/mm^2$) on day 1 ($P < 0.05$; Fig. 2C). In MDA-MB-231 tumor, MVD was significantly decreased 8 days after treatment with either E7080 ($23.3 \pm 7.2/mm^2$) or bevacizumab ($41.8 \pm 7.0/mm^2$) compared with treatment with vehicle ($81.2 \pm 6.2/mm^2$; Fig. 2B; $P < 0.05$). On the other hand, LVD was not affected by bevacizumab ($22.3 \pm 5.7/mm^2$) but decreased by E7080

Table 3. Amounts of VEGF, VEGF-C, and VEGF-D secreted into conditioned medium were determined by ELISA assays

Cell	VEGF-C	VEGF-D	VEGF
MDA-MB-231	15,321	<62.5	2,800
MDA-MB-435	<94	<62.5	2,600

NOTE: Values in table expressed as pg/mL.

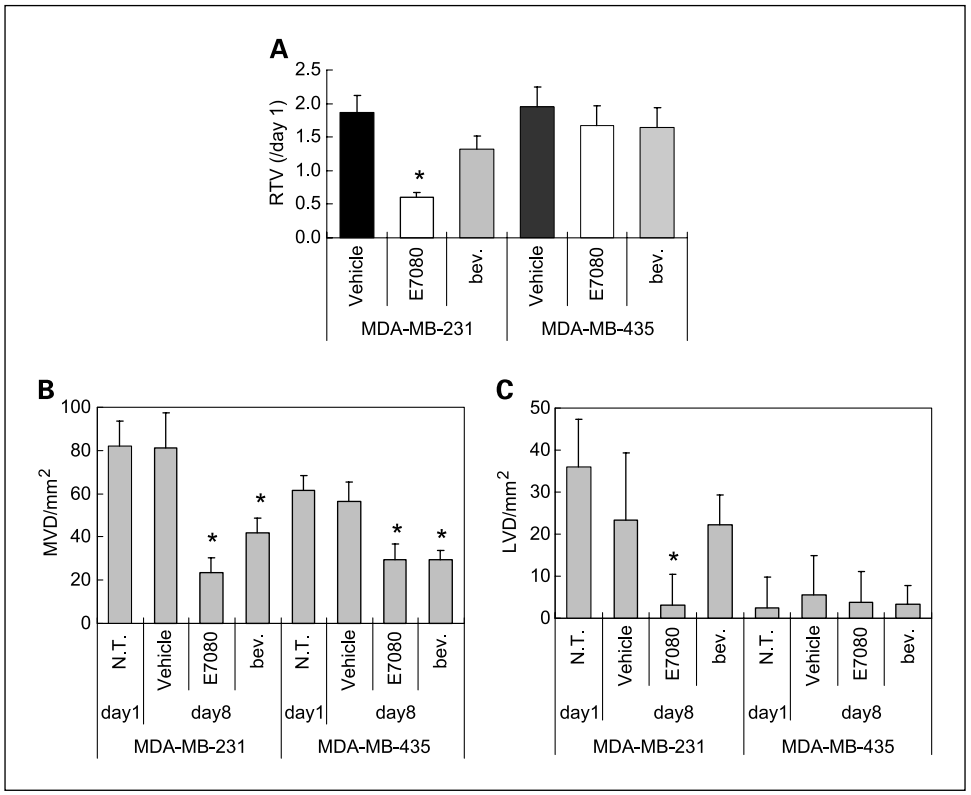

Fig. 2. Effects of E7080 on angiogenesis or lymphangiogenesis induced by human breast cancer MDA-MB-231 and MDA-MB-435 cells after m.f.p. inoculation. *A,* effect of E7080 and bevacizumab on the primary tumor growth in the m.f.p. Treatment was started when the primary tumor volume was ~180 mm^3 (day 1). E7080 was orally administered once a day for 7 d and bevacizumab was i.v. injected twice a week. Antitumor activity is shown as RTV (RTV = calculated tumor volume on day 8/tumor volume on day 1). Columns, average; bars, SD. *B,* effect of E7080 and bevacizumab on angiogenesis within the primary tumor. Angiogenesis (MVD) was determined by immunohistochemical analysis with anti-CD31 antibody. *C,* effect of E7080 and bevacizumab on lymphangiogenesis within the primary tumor. Lymphangiogenesis (LVD) was determined by immunohistochemical analysis with anti-LYVE-1 antibody. *, $P < 0.05$.

(3.2 ± 3.3/mm^2) compared with treatment with vehicle (23.2 ± 6.4/mm^2; Fig. 2C; $P < 0.05$). These data suggest that only E7080 suppresses lymphangiogenesis in MDA-MB-231 tumors (Fig. 3). In MDA-MB-435 tumor, bevacizumab and E7080 treatment decreased MVD by almost the same extent (29.4 ± 7.3 and 29.3 ± 4.3/mm^2, respectively) compared with treatment with vehicle (56.4 ± 9.2/mm^2; Fig. 2B; $P < 0.05$). Relative growth of both the MDA-MB-231 and MDA-MB-435 tumors was less than 2-fold (1.90 ± 0.4 and 1.95 ± 0.3, respectively) for 8 days (Fig. 2A). Nevertheless, E7080 significantly inhibited tumor growth of MDA-MB-231 ($P < 0.05$). Production of the fully processed form of VEGF-C by MDA-MB-231 (Supplementary Fig. S2), which can activate VEGF-R2 and is not inactivated by bevacizumab, suggested that the processed VEGF-C/VEGF-R2 signal pathway might participate in angiogenesis in MDA-MB-231 tumors (Fig. 2A). Consistent with antitumor activity, the inhibition of angiogenesis (MVD) was stronger with E7080 treatment than bevacizumab in the MDA-MB-231 model (Fig. 2B).

E7080 inhibits metastasis to both regional lymph nodes and distant lung in the MDA-MB-231 m.f.p. xenograft model. Next, we evaluated the effects of E7080 and bevacizumab on metastases of MDA-MB-231 to the regional lymph nodes and distant lung. Time to develop metastases of MDA-MB-231 was ~7 weeks. We treated tumor-bearing mice with inhibitors 43 days after inoculation and administered for 56 days (Fig. 4). Both E7080 and bevacizumab significantly inhibited local tumor growth at the m.f.p., and at the end of treatment, RTVs were 0.81 ± 1.00 (for E7080), 5.11 ± 6.54 (for bevacizumab), and 17.4 ± 13.1 (for vehicle; $P < 0.05$; Fig. 4). E7080 also

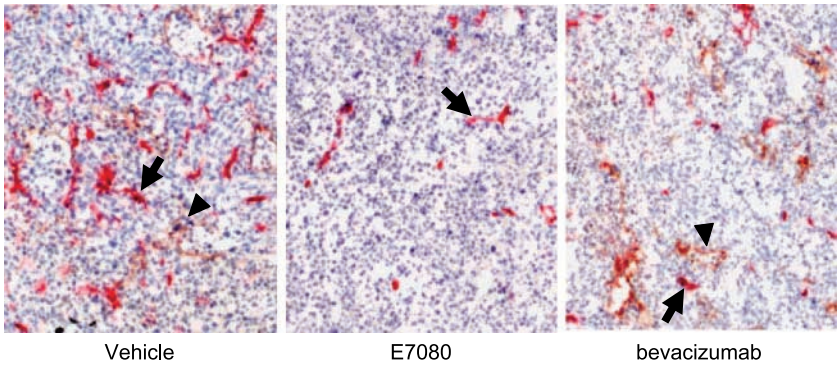

Fig. 3. Immunohistochemical analysis of angiogenesis and lymphangiogenesis within the primary MDA-MB-231 tumor in the m.f.p. Tumors were treated for 7 d and stained for an endothelial cell marker CD31 (*arrow, red*) and a lymph endothelial cell marker LYVE-1 (*arrowhead, brown*). Photographs were taken under a microscope at a magnification of ×20. Representative photographs were shown.

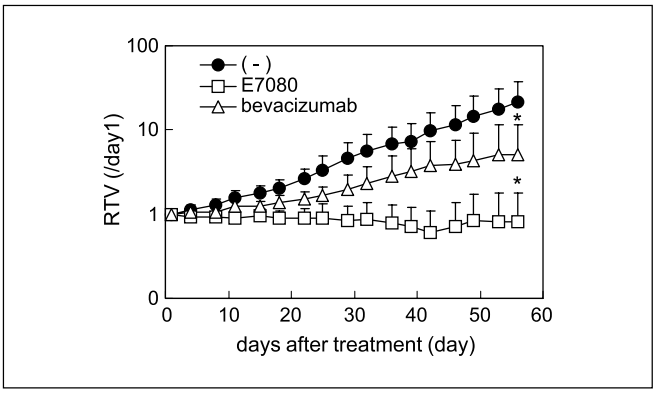

Fig. 4. Antitumor activities of E7080 and bevacizumab on the growth of primary tumor in the MDA-MB-231 xenograft model. Tumor fragments (17 ± 2 mg) were inoculated into the m.f.p. and treatment was started ~2 wk after tumor inoculation. E7080 (100 mg/kg) was orally administered once a day and bevacizumab (800 μg per mouse) was administered i.v. twice a week for 8 wk. Effects of E7080 and bevacizumab on the primary tumor growth in the m.f.p. Points, average; bars, SD.

significantly inhibited metastasis to both regional lymph nodes and distant lung ($P < 0.05$; Table 2). Metastases to lymph nodes occurred in 0 of 10 mice and to the lung in 0 of 10 mice after E7080 treatment, whereas metastases to both the lymph nodes and lung occurred in 9 of 12 vehicle-treated mice. Bevacizumab also seemed to decrease the incidence of metastases to the lymph nodes (6 of 10) and lung (3 of 10), but this decrease was only significant in the lung (Table 2). These results suggest that bevacizumab was not able to inhibit the VEGF-C/VEGF-R3 signal.

E7080 decreased both angiogenesis and lymphangiogenesis of established metastatic nodules of MDA-MB-231 tumor in the lymph nodes. We observed a significant decrease in both lymphangiogenesis and angiogenesis in the primary MDA-MB-231 tumor with E7080 treatment (Fig. 3). Thus, we evaluated the effect of E7080 on the growth of metastatic nodules, angiogenesis, and lymphangiogenesis within established metastatic nodules in the lymph nodes after resecting the primary tumor at the m.f.p. (Fig. 5A). The primary tumors were resected ~90 days after inoculation (Fig. 5A) and E7080 was administered beginning 2 weeks after tumor resection for 4 weeks (Fig. 5C). E7080 seemed to inhibit the growth of metastatic nodules (vehicle: 11.8 ± 10.8; E7080: 0.6 ± 0.3; Fig. 5B and C), but it was not a statistical difference because of large variation of RTVs in the vehicle group, although immunohistochemical analysis with anti-CD31 and anti-LYVE-1 antibody (Fig. 6) indicated that E7080 treatment significantly decreased both MVD (vehicle: 94.3 ± 12.6; E7080: 20.3 ±

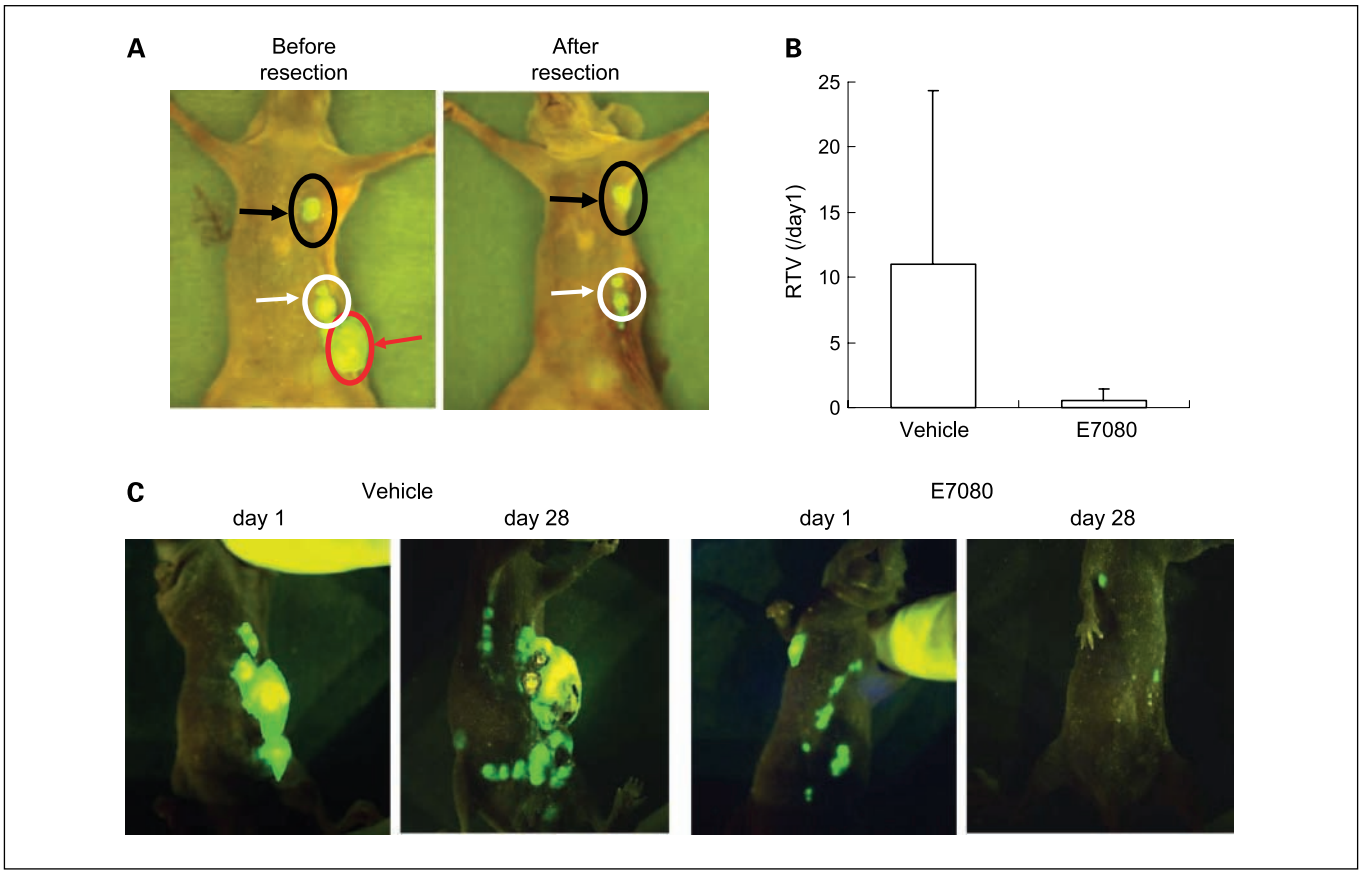

Fig. 5. Effect of E7080 on the growth of metastatic nodules of MDA-MB-231 in the lymph nodes after resection of the primary tumor. *A,* photographs of mice after resection of the primary tumor. Tumor cells were inoculated in the m.f.p., and ~90 d later, the primary tumors were resected. Red arrow, primary tumor; white arrows, metastasis to the skin lymph nodes; black arrows, metastasis to the axillary lymph nodes. *B,* effect of E7080 on the growth of metastatic nodules in the lymph nodes. E7080 (100 mg/kg) was administered orally once a day from day 1 to day 28 after resection of the primary tumors. Columns, antitumor activity shown as a RTV (RTV = tumor volume on day 28/tumor volume on day 1) as the average; bars, SD. *C,* photographs of mice bearing metastatic nodules in the lymph nodes treated with either vehicle or E7080 on days 0 and 28. Representative photographs were shown.

2.9/mm^2; Fig. 6A and C) and LVD (vehicle: 24.7 ± 13.3; E7080: 1.0 ± 0.9/mm^2; Fig. 6B and C) within metastatic nodules in the lymph nodes. These results showed that E7080 inhibited both angiogenesis and lymphangiogenesis within established metastatic nodules in lymph nodes in this MDA-MB-231 xenograft model.

Discussion

A novel multikinase inhibitor, E7080, was an equally potent inhibitor of VEGF-R2 and VEGF-R3 kinases in both cell-free and cell-based assays. Oral administration of E7080 decreased angiogenesis and lymphangiogenesis as determined by MVD and LVD, respectively, in the primary tumor in MDA-MB-231 m.f.p. xenograft models. E7080 also decreased MVD and LVD within metastatic nodules in the lymph nodes in nude mice.

In m.f.p. xenograft models of VEGF-C–overexpressing MDA-MB-435 cells, the VEGF-C/VEGF-R3 signal played a role in tumor cell dissemination from the primary tumor by inducing lymphangiogenesis, resulting in the promotion of metastases to regional lymph nodes and distant lung metastases (18). Suppression of VEGF-C–promoted metastases to both sites was more effectively inhibited by anti-VEGF-R3 antibody than by anti-VEGF-R2 antibody when antibodies were administered before the development of micrometastasis. Treatment with anti-VEGF-R3 antibody following the development of micrometastasis was less effective compared with early treatment. Because E7080 almost completely inhibited MDA-MB-231 metastases to distant lung in our model, E7080 might suppress dissemination of MDA-MB-231 cells based on lymphangiogenesis inhibition in addition to growth inhibition of metastatic nodules in the lung based on angiogenesis inhibition.

The VEGF-C/VEGF-R3 signal directly promoted invasion of cancer cells and increased both lymph node and lung metastases of human lung adenocarcinoma cells in mice (26). Treatment with a soluble form of VEGF-R3 (Flt4/Fc) suppressed lung and lymph node metastases of two distinct lung tumors (A549 cells and VEGF-C–overexpressing H928 cells), although Flt4/Fc did not affect the growth of primary tumors. The VEGF-C/VEGF-R3 signal may have a role in invasion of tumor cells associated with distant metastasis. VEGF-C confers an ability to metastasize to both lymph nodes and the lung in MDA-MB-435 and H928 tumors (18, 26). However, VEGF-C–overexpressing MDA-MB-435 cells developed only micrometastases with less vasculature at both sites. Unlike MDA-MB-435 cells, VEGF-C–overexpressing H928 cells developed macroscopic metastases characterized by extensive proliferation in metastatic sites. Therefore, VEGF-C overexpression alone is not enough to assure the growth of metastatic nodules. The combination treatment against VEGF-R2 and anti-VEGF-R3 is more effective against lymph node and lung metastases than treatment with anti-VEGF-R2 antibody alone (18). Dual inhibition of both VEGF-R2 and VEGF-R3 was a better strategy to suppress metastases of VEGF-C–overexpressing tumors. Consistent with this, E7080 significantly inhibited both lymph nodes and lung metastasis in our MDA-MB-231 m.f.p. models, whereas bevacizumab significantly inhibited lung metastases only.

Roberts et al. (18) reported that VEGF-C overexpressed in MDA-MB-435/GFP cells promoted tumor metastases to lymph nodes and anti-VEGF-R3 antibody inhibited VEGF-C–promoted metastases (18). This report suggested that

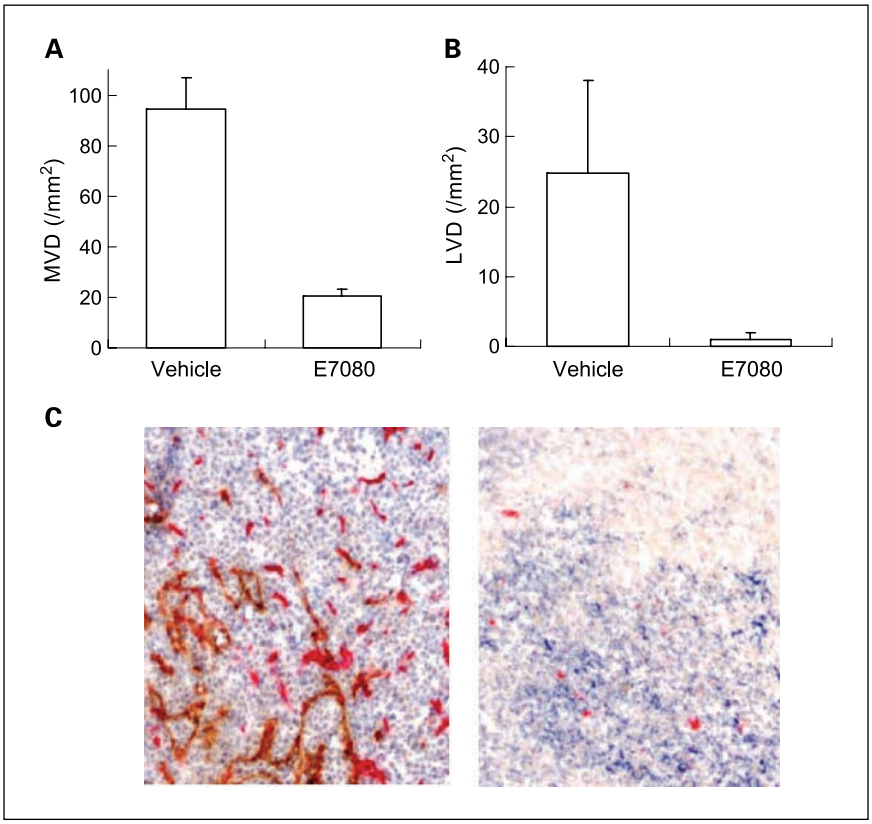

Fig. 6. Effects of E7080 on either angiogenesis or lymphangiogenesis within the metastatic nodules of MDA-MB-231 tumor in the lymph nodes. E7080 (100 mg/kg) was administered orally once a day from day 1 to day 28 after resection of the primary tumors. *A,* effect of E7080 on angiogenesis. Columns, average; bars, SD. *B,* effect of E7080 on lymphangiogenesis. Columns, average; bars, SD. *C,* immunohistochemical analysis of angiogenesis and lymphangiogenesis within the metastatic nodules in the lymph nodes. Angiogenesis was determined by immunohistochemical analysis with anti-CD31 antibody (*red*, fuchsin-alkaline phosphatase) and lymphangiogenesis was determined by immunohistochemical analysis with anti-LYVE-1 antibody (*brown*, horseradish peroxidase-3,3′-diaminobenzidine). Photographs were taken under a microscope at a magnification of ×20. Representative photographs were shown.

MDA-MB-435 cells did not metastasize to lymph nodes because of lack of VEGF-C expression, although they expressed enough amount of VEGF to develop lung metastasis. Rae et al. (27) clearly showed the misidentification of the MDA-MB-435 cell line as a breast cancer cell line. In this article, we focused on distinguishing the role of VEGF-C/VEGF-R3 signaling in lymphangiogenesis and lymph node metastasis from the role of VEGF/VEGF-R2 signaling in angiogenesis and lung metastasis. Therefore, MDA-MB-435 might be a suitable control tumor cell line to compare MDA-MB-231 cells.

DC101, which was the inhibitory antibody against VEGF-R2, inhibited lymphangiogenesis within the primary tumor of VEGF-C–overexpressing MDA-MB-435 cells, suggesting that VEGF-R2 expressed in lymphatic endothelial cells also participated in tumor lymphangiogenesis (18). Because VEGF-R3 expression in lymph endothelial cells is regulated by angiopoietin-2, which is up-regulated by VEGF, VEGF might indirectly participate in lymphangiogenesis (28). Bevacizumab tended to decrease lymph node metastasis of MDA-MB-231 and it might be dependent on a reduction of VEGF-R3 in lymph endothelial cells.

The VEGF-C/VEGF-R3 signal plays several roles in tumor lymphangiogenesis and metastasis: (*a*) induction of lymphangiogenesis by VEGF-C within the primary and metastatic tumors and promotion of metastasis by dissemination of tumor cells via newly formed lymphatic vessels; (*b*) induction of invasive activity of some types of tumors, in which VEGF-R3 is expressed; and (*c*) induction of lymphangiogenesis by VEGF via activation of VEGF-R2 expressed on lymph endothelial cells. Among several multiple kinase inhibitors we tested, E7080 was one of the most potent dual inhibitors of VEGF-R2 and VEGF-R3 kinases in cell-free kinase assays (Supplementary Fig. S3) and E7080 showed stronger inhibitory activity than sunitinib with cell phosphorylation assay using HUVEC (Supplementary Fig. S4). E7080 may show more potent antilymphangiogenic and antiangiogenic activity compared with similar inhibitors of the same class of drugs. E7080 effectively prevented regional lymph node metastases and their further growth. Thus, inhibition of both VEGF/VEGF-R2 and VEGF-C/VEGF-R3 signals with E7080 is a promising new approach to inhibit development of metastases of tumors overexpressing VEGF-C.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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