

E7080 Suppresses Hematogenous Multiple Organ Metastases of Lung Cancer Cells with Nonmutated Epidermal Growth Factor Receptor

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Abstract

While epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors improve the prognosis of patients with *EGFR* mutant lung cancer, the prognosis of patients with nonmutant *EGFR* lung cancer, especially those with metastases, is still extremely poor. We have assessed the therapeutic efficacy of E7080, an orally available inhibitor of multiple tyrosine kinases including VEGF receptor 2 (VEGFR-2) and VEGFR-3, in experimental multiple organ metastasis of lung cancer cell lines without *EGFR* mutations. E7080 markedly inhibited the *in vitro* proliferation of VEGF-stimulated microvascular endothelial cells. Intravenous inoculation into natural killer cell-depleted severe combined immunodeficient mice of the small cell lung cancer cell lines H1048 (producing low amounts of VEGF) and SBC-5 (producing intermediate amounts of VEGF) resulted in hematogenous metastases into multiple organs, including the liver, lungs, kidneys, and bones, whereas intravenous inoculation of PC14PE6, a non-small cell lung cancer cell line producing high amounts of VEGF, resulted in lung metastases followed by massive pleural effusion. Daily treatment with E7080 started after the establishment of micrometastases significantly reduced the number of large (>2 mm) metastatic nodules and the amount of pleural effusion, and prolonged mouse survival. Histologically, E7080 treatment reduced the numbers of endothelial and lymph endothelial cells and proliferating tumor cells and increased the number of apoptotic cells in metastatic nodules. These results suggest that E7080 has antiangiogenic and antilymphangiogenic activity and may be of potential therapeutic value in patients with nonmutant *EGFR* lung cancer and multiple organ metastases. *Mol Cancer Ther*; 10(7); 1218–28. ©2011 AACR.

Introduction

Lung cancer is one of the most prevalent malignancies and the leading cause of cancer-related deaths worldwide (1). The high mortality of this disease is predominantly due to the high metastatic potential of lung cancer. Although platinum-based cytotoxic chemotherapy is

standard treatment for both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) with metastases (2), the median survival of these patients is only about 12 to 14 months. Recently, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have been shown to prolong progression-free survival of lung cancer patients with tumors containing mutated *EGFR*, extending the median survival of these patients to 22 to 30 months (3, 4). Nevertheless, the prognosis of patients with metastatic SCLC and NSCLC with nonmutant *EGFR* lung cancer has not yet been improved (5).

Angiogenesis is essential for tumor enlargement and metastasis and is regulated by proangiogenic and antiangiogenic molecules (6). VEGF-related molecules (VEGF, VEGF-B, VEGF-C, VEGF-D, and placental growth factor) and receptors (VEGFR-1, VEGFR-2, and VEGFR-3) play pivotal roles in angiogenesis and lymphangiogenesis (7). Binding of VEGF to VEGFR-2 is the critical signal for tumor angiogenesis, as well as inducing vascular hyperpermeability and promoting the production of pleural effusion and ascites (8, 9). VEGF-C and VEGF-D activate VEGFR-3 and are considered lymphangiogenic factors, although the fully processed form of VEGF-C also

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activates VEGFR-2 (10). In addition, the binding of VEGF-C to VEGFR-3 has been found to promote intratumoral lymphangiogenesis and lymph node metastasis in a preclinical study (11).

The anti-VEGF monoclonal antibody, bevacizumab, has been used successfully to treat patients with several malignant diseases including nonsquamous NSCLC (12, 13). However, the combination of bevacizumab and cytotoxic chemotherapy has been found to have marginal effects on patient survival (12, 14), suggesting the need for novel therapeutic modalities.

E7080 is an orally active inhibitor of VEGFR-2 and VEGFR-3, with additional activity against other receptor tyrosine kinases, including fibroblast growth factor receptors (FGFR), platelet-derived growth factor receptors (PDGFR), and c-Kit (15). E7080 shows potent anti-tumor effects in xenograft models of various types of tumors by inhibiting angiogenesis, especially through VEGFR-2/3 suppression (16, 17). We have tested the therapeutic efficacy of E7080 against lung cancer cell lines expressing wild-type EGFR, using *in vivo* experimental metastasis models.

Materials and Methods

Cell lines

The human SCLC cell line SBC-5 and a human NSCLC cell line PC14PE6 were kindly provided by Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan; ref. 18) in 2006 and Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX; ref. 9) in 1999, respectively. The human SCLC cell line H1048 and human NSCLC cell line PC-9 were purchased from American Type Culture Collection in 2004 and Immuno-Biological Laboratories Co.; ref. 19) in 2007, respectively. Flow cytometric analyses revealed that H1048, PC14PE6, and SBC-5 cells showed high, intermediate, and very low wild-type EGFR protein expression, respectively (data not shown). SBC-5 cells were maintained in Eagle's Minimum Essential Media (MEM) and H1048, PC14PE6, and PC-9 cells were maintained in RPMI 1640 medium, each supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (50 µg/mL). Human dermal microvascular endothelial cells (HMVEC) were purchased from KURABO in 2007 and maintained in HuMedia-MvG with growth supplements (KURABO). All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for *Mycoplasma* with the use of a MycoAlert Mycoplasma Detection Kit (Lonza). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

Reagents

4-[3-Chloro-4-(*N'*-cyclopropylureido)phenoxy]-7-methoxyquinoline-6-carboxamide (E7080) was synthesized by Eisai Co. Ltd., as described (15, 16). For *in vitro* experiments, a stock solution of E7080 (10 mmol/L)

was prepared in dimethyl sulfoxide, stored at -20°C, and diluted with culture media before use. For *in vivo* experiments, E7080 was dissolved in distilled water and stored until use at 4°C. Recombinant human VEGF165 and recombinant human basic fibroblast growth factor (bFGF) were purchased from R&D Systems. An anti-mouse interleukin 2 receptor β-chain monoclonal antibody, TM-β1 (IgG2b), was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan; ref. 20).

Expression of VEGF, VEGF-C, VEGF-D, and VEGFR mRNAs

Expression of VEGF, VEGF-C, VEGF-D, and VEGFR mRNAs was measured by reverse transcriptase (RT) PCR. Total cellular RNA was isolated using RNeasy Mini kits and RNase-free DNase kits (Qiagen) according to the manufacturer's protocols. Total RNAs were reversely transcribed using an Omniscript RT kit (Qiagen). PCRs were carried out using Ex Taq Hot Start Version (Takara) and the primers are shown in Supplementary Table S1. RT-PCR products were electrophoresed on agarose gels, and the bands were visualized by ethidium bromide staining.

VEGF production

Tumor cells (1×10^6) were cultured in RPMI 1640 medium with 10% FBS for 24 hours. The cells were washed with PBS and incubated for 48 hours in RPMI 1640 medium with 10% FBS. The culture medium was harvested and centrifuged, and the supernatants were stored at -70°C until analysis. VEGF concentration was assayed by ELISA as described by the manufacturer (R&D Systems). All samples were run in triplicate, and each assay was conducted 3 times independently.

Flow cytometric analysis

HMVECs and tumor cells were harvested and resuspended in PBS. After 2 washes with PBS, the cells were incubated for 45 minutes at 4°C with phycoerythrin (PE)-labeled anti VEGFR-1, VEGFR-2, and VEGFR-3 antibodies or with PE-labeled mouse IgG1 antibody. The intensity of fluorescence was measured by flow cytometric analysis using FACScan (Becton Dickinson).

Cell proliferation assay

Cell proliferation was measured using the MTT dye reduction method (21). Briefly, tumor cells (2×10^3 cells per 100 µL per well), plated in triplicate in 96-well plates, were incubated in medium containing 10% FBS for 24 hours. HMVECs (5×10^3 cells per 100 µL per well), plated in triplicate in 96-well plates precoated with 1.5% gelatin, were incubated in Eagle's MEM containing 5% FBS for 24 hours. Tumor cells were incubated with several concentrations of E7080 for a further 72 hours. HMVECs were incubated for 72 hours with E7080 in the presence or absence of VEGF or bFGF. To each well was added 50 µL of MTT solution (2 mg/mL; Sigma), followed by incubation for a further 2 hours. The media containing

MTT solution were removed, and the dark blue crystals in each well were dissolved in 100 μ L dimethyl sulfoxide. The absorbance was measured at test and reference wavelengths of 550 and 630 nm, respectively.

To determine the bioactivity of VEGF produced by the tumor cells, WST assays were conducted using Cell Counting Kit-8 (Dojindo). Briefly, HMVECs (5×10^3 cells per 100 μ L per well) were plated in triplicate in 96-well plates precoated with 1.5% gelatin. After 24 hours, HMVECs were incubated with cell culture supernatants of tumor cells in the presence or absence of E7080 (10 nmol/L) for 72 hours. Following the addition of 10 μ L of WST-8 reagents, the cells were incubated for a further 2 hours, and absorbance was measured at 450 nm and 630 nm.

Western blot analysis

HMVECs were cultured until confluence in EBM medium containing 0.5% FBS for 24 hours. Cells were treated with E7080 at indicated concentrations for 120 minutes, stimulated with VEGF165 (20 ng/mL) for 10 minutes, and collected in Cell Lysis Buffer (Cell Signaling Technology) containing 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology). For Western blot analysis, 20 μ g of total protein was resolved by SDS-PAGE (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Atto). After 3 washes, the membranes were incubated with Blocking One (Nacalai Tesque Inc.) for 1 hour at room temperature and incubated overnight at 4°C with 1:1,000 dilutions of primary antibodies to p-VEGFR-2 (Tyr 996; Cell Signaling Technology), VEGFR-2 (C-1158; Santa Cruz Biotechnology), phospho-p44/p42 MAPK (Thr202/Tyr204; Cell Signaling Technology), and p44/p42 MAPK (Cell Signaling Technology). After washing, the membranes were incubated for 2 hours at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescent substrate (Pierce Biotechnology).

Animals

Male severe combined immunodeficient mice (SCID) mice and athymic BALB/c nude mice, 5 to 6 weeks old, were obtained from CLEA Japan and maintained under specific pathogen-free conditions throughout this study. All experiments were carried out in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

In vivo metastasis models

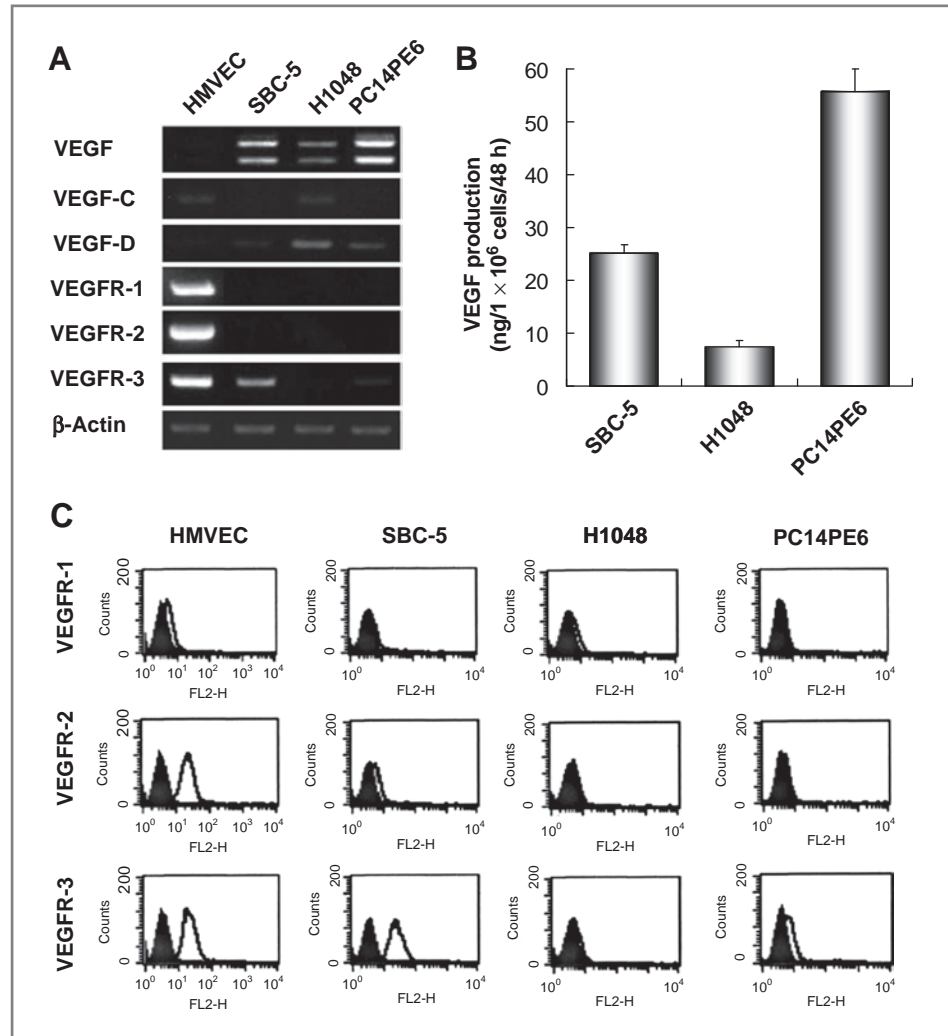
To facilitate metastasis formation, SCID mice were pretreated with anti-mouse interleukin 2 receptor β -chain antibody to deplete natural killer (NK) cells (20). Two days later, the mice were inoculated with SBC-5 or H1048 cells (1.0×10^6 per mouse) into the tail vein. Nude mice were intravenously inoculated via the tail vein with PC14PE6 cells (1.0×10^6 per mouse). As

we reported previously, micrometastases were detected as early as 14 days after tumor cell inoculation (9). To evaluate the therapeutic efficacy of E7080 against established metastatic nodules, the mice were treated once daily with 1, 3, and 10 mg/kg/d E7080 by oral gavage, beginning 2 (SBC-5 and PC14PE6 cells) or 4 weeks (H1048 cells) after inoculation. Five (SBC-5 and PC14PE6 cells) or 8 weeks (H1048 cells) after tumor cell inoculation, the mice were anesthetized by intraperitoneal injection of pentobarbital, and radiographs were taken to determine bone metastases. The mice were killed humanely under anesthesia and the major organs were removed and weighed. The lungs were fixed in Bouin's solution (Sigma) for 24 hours. The number of metastatic colonies on the surface of the organs and the number of osteolytic lesions evident in radiographs were counted by 2 investigators independently (H. Ogino and M. Hanibuchi). To determine the effect of E7080 on survival, mice (10 per group) were treated once daily with 10 mg/kg/d E7080 from day 14 until they became moribund.

Immunohistochemical-immunofluorescent determination of endothelial cells, proliferating and apoptotic tumor cells, and VEGF production

Major organs containing metastases were fixed in 10% formalin and embedded in paraffin, or TissueTek optimum cutting temperature medium (Sakura) and frozen immediately. The paraffin-embedded tissues were used to quantitate *in vivo* cell proliferation using mouse anti-human Ki-67 mAb (MIB1; 1:50 dilution; DAKO), apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method with the Apoptosis Detection System (Promega), and VEGF production using mouse anti-human VEGF mAb (1:100 dilution; Pharmingen). To detect endothelial cells, frozen tissue sections (10 μ m thick) were fixed with cold acetone and incubated with rat anti-mouse CD31/PECAM-1 monoclonal antibody (1:100 dilution; Pharmingen). To detect lymph endothelial cells, frozen sections were fixed with midl form and incubated with rabbit anti-mouse LYVE-1 monoclonal antibody (1:100 dilution; Abcam). Appropriate secondary antibodies conjugated with peroxidase and the 3,3'-diaminobenzidine tetrahydrochloride (DAB) Liquid System (DakoCytomation) was used to detect immunostaining. To detect CD31 and LYVE-1 simultaneously, frozen sections were fixed with midl form for 1 minute and incubated with rat anti-mouse CD31 and rabbit anti-mouse LYVE-1 antibodies, followed by incubation with anti-rat IgG conjugated to Alexa488 (green) and anti-rabbit IgG conjugated to Alexa594 (red; 1:100 dilution; Molecular Probes). In each analysis, the 5 areas containing the highest number of stained cells within a section were selected for histologic quantification under light microscopy or fluorescent microscopy with a 200-fold magnification. The results were independently evaluated by 2 investigators (H. Ogino and M. Hanibuchi).

Figure 1. *In vitro* production of VEGF by lung cancer cell lines bearing nonmutant EGFR. A, expression of mRNAs encoding members of the VEGF and VEGFR families in human lung cancer cell lines and endothelial cells (HMVEC) bearing nonmutant EGFR as determined by RT-PCR. B, VEGF production by lung cancer cells bearing nonmutant EGFR as determined by ELISA. C, expression of VEGFRs protein by HMVECs and lung cancer cell lines bearing nonmutant EGFR as determined by flow cytometry.



Statistical analysis

Differences were analyzed by 1-way ANOVA, followed when appropriate by Newman-Keuls multiple comparison tests. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using the GraphPad Prism Program Ver. 4.01.

Results

Expression of VEGF, VEGF-C, VEGF-D, and their receptors in human endothelial cells and lung cancer cell lines *in vitro*

We determined the expression of VEGF, VEGF-C, VEGF-D, and their receptors in HMVECs and 3 lung cancer cell lines at both the mRNA and protein levels. PC14PE6, SBC-5, and H1048 cells showed high, intermediate, and low VEGF expression, respectively (Fig. 1A and B). Although VEGFR-1 and VEGFR-2 were expressed only in HMVECs, both SBC-5 cells and HMVECs expressed VEGFR-3 mRNA and protein (Fig. 1A and C). However, we could not detect the production of its

ligands, VEGF-C and VEGF-D, in the 3 lung cancer cell lines by ELISA (data not shown). Although these cell lines expressed mRNA of several receptors, including FGFR-1, FGFR-2, PDGFR- α , PDGFR- β , and c-kit, the level was generally very low (Supplementary Fig. S1). We also examined the production of ligands for FGFRs (acidic FGF and bFGF), PDGFRs (PDGF-AA, PDGF-AB, and PDGF-BB), and c-Kit (stem cell factor). The levels of these ligands were much lower than that of VEGF (data not shown).

E7080 inhibition of VEGF-induced proliferation and VEGFR-2 phosphorylation of endothelial cells, but not tumor cells, *in vitro*

Chemical structure of E7080 was shown in Fig. 2A. When we assayed the effect of the multiple tyrosine kinase inhibitor, E7080, on the proliferation of tumor cells and endothelial cells *in vitro*, we found that E7080 did not inhibit the proliferation of the 3 human lung cancer cells ($IC_{50} > 1,000$ nmol/L; Fig. 2B). In contrast, low-dose E7080 suppressed the proliferation of VEGF-stimulated HMVECs

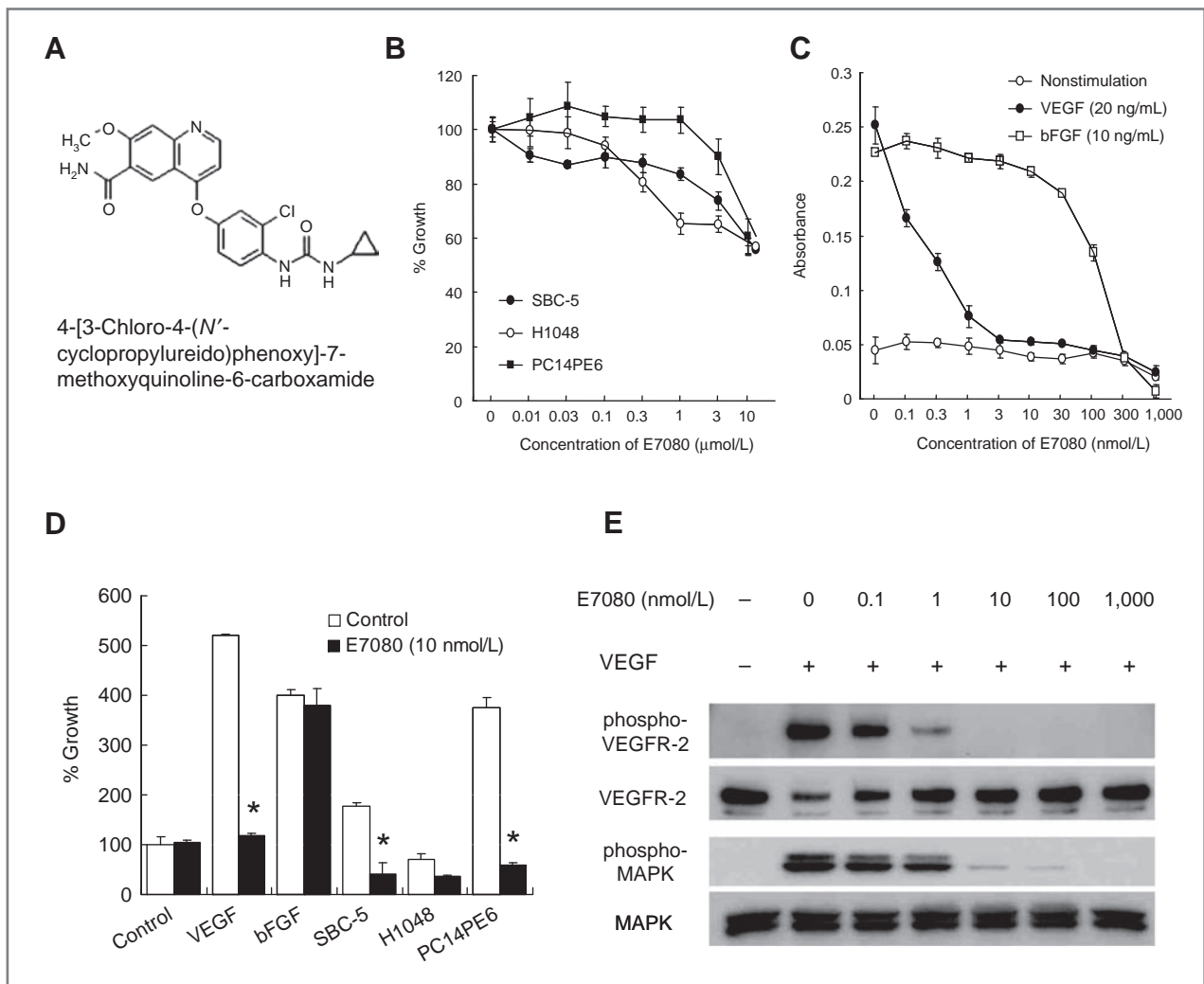


Figure 2. E7080 inhibition of VEGF-induced endothelial proliferation via inhibition of VEGFR-2 phosphorylation. **A**, chemical structure of E7080. **B–D**, effect of E7080 on the proliferation of lung cancer cell lines bearing nonmutant EGFR (**B**), human endothelial cells (HMVEC) stimulated with recombinant VEGF and bFGF (**C**), and HMVECs stimulated with culture medium from lung cancer cell lines bearing nonmutant EGFR (**D**) as determined by MTT assays. **E**, effect of E7080 on VEGFR-2 phosphorylation as determined by Western blot analysis.

($\text{IC}_{50} = 0.3 \text{ nmol/L}$), whereas high-dose E7080 suppressed the proliferation of bFGF-stimulated HMVECs ($\text{IC}_{50} = 100 \text{ nmol/L}$; Fig. 2C). The culture supernatants of SBC-5 and PC14PE6 cells stimulated HMVEC proliferation, effects abrogated by 10 nmol/L E7080, a dose sufficient to inhibit the proliferation of HMVECs induced by VEGF but not by bFGF (Fig. 2D). These results suggest that VEGF but not bFGF may be the predominant tumor cell-derived growth factor for HMVEC produced by these lung cancer cell lines.

E7080 also dose dependently suppressed the VEGF-induced VEGFR-2 phosphorylation and activation of downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, in HMVECs (Fig. 2E), suggesting that E7080 has potent activity against VEGF-induced angiogenesis. We also confirmed that E7080 inhibited VEGF-C-induced phosphorylation of

VEGFR-3 (Supplementary Fig. S2), in agreement with previous results (16).

E7080 suppression of enlargement of metastases of lung cancer cells expressing nonmutant EGFR in immunodeficient mice

We next examined the effect of E7080 on metastasis induced by lung cancer cells expressing nonmutant EGFR. Intravenous inoculation of the SCLC cell line, SBC-5, into NK cell-depleted SCID mice has been shown to produce experimental metastases within 35 days (22–24). These metastatic lesions were located primarily in the liver where some were greater than 2 mm in diameter. In addition, SBC-5 cells produce lung metastases (<2 mm in diameter) and osteolytic bone metastases detectable by radiography (Fig. 3 and Table 1). Treatment with E7080

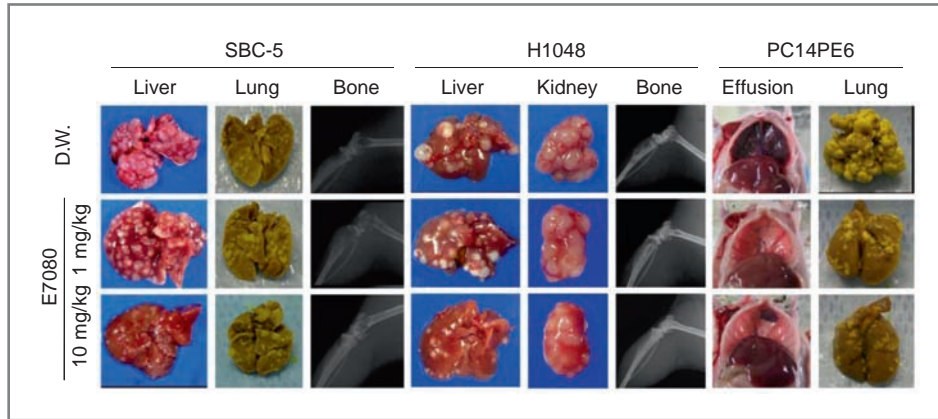


Figure 3. E7080 inhibition of the enlargement of hematogenous multiple organ metastases of lung cancer cells bearing nonmutant *EGFR* in immunodeficient mice. SBC-5 and H1048 cells were inoculated intravenously into NK cell-depleted SCID mice, whereas PC14PE6 cells were intravenously inoculated into nude mice. Mice received oral E7080 on days 14 to 35 after inoculation with SBC-5 or PC14PE6 cells and on days 28 to 56 after inoculation with H1048 cells. Representative pictures of 5 to 8 mice per group are shown. D.W., distilled water.

reduced significantly the number of large liver metastases (>2 mm in diameter) and total liver weight even at low doses (1 mg/kg/d) but did not reduce the total number of liver metastases (Fig. 3 and Table 1). At higher doses (3 and 10 mg/kg/d), E7080 also significantly reduced the number of osteolytic bone lesions but, interestingly, did not affect the number of lung metastases. E7080 treatment did not cause apparent adverse events, such as loss of body weight (data not shown).

We found that a second SCLC cell line, H1048, also produced metastatic nodules in multiple organs of NK cell-depleted SCID mice, although its pattern of metastases differed from that of SBC-5 cells. H1048 cells produced nodules in the liver, kidneys, and bones (Fig. 3). Once daily treatment with high dose (10 mg/kg/d), but not low dose (1 mg/kg/d), E7080 suppressed the number of large metastases in the liver and kidneys, as well as reducing the total weight of the liver and kidney and the number of metastatic nodules in bone (Fig. 3 and Table 1).

In contrast, human lung adenocarcinoma cells, PC14PE6, produced large colonies only in the lung and induced large volumes of pleural effusion in nude mice (Fig. 3 and Table 1; refs. 9, 25). Treatment with E7080 reduced significantly the number of large lung metastases and total lung weight even at low doses (1 mg/kg/d), as well as completely suppressing the production of pleural effusion (Fig. 3 and Table 1). These results suggest that E7080 may prevent the enlargement of metastatic colonies in multiple organs including the lungs.

E7080 inhibition of the growth of macroscopically detectable metastatic nodules

We previously reported that intravenously inoculated SBC-5 cells produce micrometastases and macroscopically detectable metastatic nodules within 14 and 21 days, respectively (22). We therefore determined whether treatment with E7080 could inhibit the growth of macroscopically detectable metastases. Treatment of E7080, started

on day 14 or 21, inhibited the numbers of bone and liver metastases larger than 2 mm in diameter (Supplementary Table S2), indicating that E7080 has therapeutic activity against not only micrometastases but macroscopically detectable metastases.

E7080 inhibition of angiogenesis and lymphangiogenesis in metastatic nodules

Histologic analysis showed that treatment with E7080 caused necrosis in liver metastases produced by SBC-5 cells (Fig. 4A). Because E7080 has activity against VEGFR-2 and VEGFR-3, which are crucial for angiogenesis and lymphangiogenesis, we evaluated the effects of E7080 on angiogenesis and lymphangiogenesis, as well as on proliferating and apoptotic cells in the metastases. CD31-positive cells, presumably representing endothelial cells, were detected diffusely in metastatic nodules, whereas LYVE-1-positive cells, presumably representing lymph endothelial cells, were detected predominantly in the periphery of the nodules (Fig. 4A). Double staining for CD31 and LYVE-1 showed that a small population of cells in the periphery was doubly positive for both (Fig. 4B), consistent with previous findings (26, 27). Importantly, E7080 treatment dramatically inhibited the numbers of CD31- and LYVE-1-positive cells (Fig. 4A and C). Moreover, E7080 treatment decreased the number of Ki-67-positive proliferating tumor cells and increased the number of TUNEL-positive apoptotic cells in liver metastases (Fig. 4A and C). There was no discernible difference in VEGF production between control and E7080-treated tumors (Supplementary Fig. S3). These results indicate that E7080 can inhibit both angiogenesis and lymphangiogenesis and therefore suppress the growth of metastases.

E7080 prolongation of the survival of mice bearing metastases of nonmutant EGFR lung cancer cells

We finally determined whether E7080 could prolong the survival of mice bearing metastases. SBC-5 and

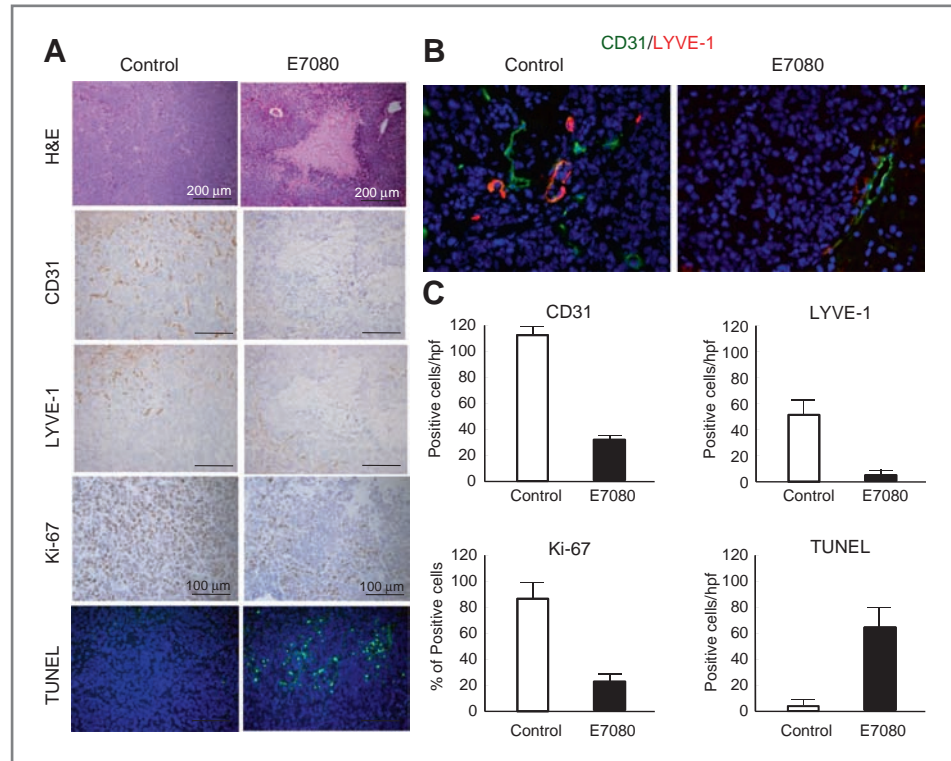
Table 1. Therapeutic effect of oral treatment with E7080 on the multiple organ metastasis produced by non-EGFR mutant lung cancer cell lines in immunodeficient mice

| Cell line | Treatment | Liver | | Lung | | Kidney | | Bone | | |
|-----------------|-----------|----------------------------|---|----------------------------|--|-------------------------|---------------------------------------|-------------------|-------------------------|----------------------|
| | | Weight, g | No. of metastasis All nodules >2 mm ^a | Weight, mg | No. of metastasis All nodules >2 mm | Weight, mg | No. of metastasis All nodules 2 mm | No. of metastasis | No. of metastasis | |
| | | | | | | | | | | |
| SBC-5 (N = 5) | Control | 2.0 (1.1–2.7) ^b | 89 (58–95) | 195 (162–212) | 66 (46–101) | All 0 | N/A | 11 (8–11) | | |
| | E7080 | | | | | | | | | |
| | 1 mg/kg | 1.1 (0.9–1.4) ^c | 59 (29–78) | 189 (176–204) | 50 (37–85) | All 0 | | 11 (8–11) | | |
| | 3 mg/kg | 1.1 (0.8–1.3) ^c | 50 (30–56) | 222 (140–276) | 66 (41–89) | All 0 | | 6 (5–7) | | |
| PC14PE6 (N = 8) | 10 mg/kg | 1.1 (1.0–1.4) ^c | 71 (30–107) | 190 (183–206) | 70 (46–85) | All 0 | | 6 (5–7) | | |
| | Control | N/A | | 558 (477–740) | 86 (85–108) | 65 (54–76) | N/A | N/A | | |
| | E7080 | | | | | | | | | |
| H1048 (N = 8) | 1 mg/kg | | | 240 (208–311) ^c | 77 (42–91) | 20 (8–41) ^c | | | | |
| | 3 mg/kg | | | 260 (206–285) ^c | 79 (56–86) | 19 (10–24) ^c | | | | |
| | 10 mg/kg | | | 205 (195–232) ^c | 69 (26–113) | 11 (2–29) ^c | | | | |
| | Control | 1.6 (1.0–2.3) | 77 (2–113) | N/A | | | 490 (300–1,020) | 52 (18–85) | 40 (19–77) | 6 (3–10) |
| E7080 | 1 mg/kg | 1.3 (1.0–1.7) | 59 (17–122) | | | | 485 (310–670) | 67 (52–83) | 45 (33–63) | 7 (4–9) |
| | 10 mg/kg | 0.9 (0.7–1.2) | 33 (3–91) | | | | 245 (200–450) ^c | 47 (27–69) | 15 (10–53) ^c | 4 (0–6) ^c |

Abbreviation: N/A, not applicable.

^aNumber of metastatic nodules larger than 2 mm in diameter.^bMedian (range).^cP < 0.05.

Figure 4. E7080 inhibition of angiogenesis and lymphangiogenesis in liver metastases of SBC-5 cells. **A**, liver metastatic nodules produced by SBC-5 cells with or without E7080 (10 mg/kg) treatment were stained for hematoxylin and eosin (H&E), endothelial cells (CD31), lymph endothelial cells (LYVE-1), proliferating human tumor cells (Ki-67), and apoptotic cells (TUNEL). Bars indicate 200 μ m for H&E, CD31, and LYVE-1, and 100 μ m for Ki-67 and TUNEL. **B**, the same nodules were double stained for CD31 (red) and TUNEL (green) to detect apoptotic endothelial cells. **C**, number of cells positive for CD31, LYVE-1, Ki-67, and TUNEL in 5 areas ($\times 200$). Bars indicate SDs.



PC14PE6 cells were intravenously inoculated into NK cell-depleted SCID and nude mice, respectively, and, beginning 14 days later, the mice were treated with E7080 (10 mg/kg). While all SBC-5-bearing mice treated with distilled water died within 40 days, treatment with E7080 significantly prolonged survival (median survival: 35 vs. 42 days; $P = 0.0001$; Fig. 5). Similarly, all PC14PE6-bearing mice treated with distilled water died within 42 days, whereas treatment with E7080 significantly prolonged survival (median survival: 37 vs. 65 days; $P = 0.0001$). These results clearly indicate that E7080 treatment has survival benefit in mice bearing metastases of nonmutant *EGFR* lung cancer cells.

Discussion

Lung cancer has been subdivided clinically into SCLC and NSCLC because these 2 subgroups have quite different clinical characteristics, such as speed of growth/spread and responses to cytotoxic chemotherapy and radiotherapy (2). The prognosis of patients with lung tumors bearing *EGFR* mutations has been markedly improved by treatment with the *EGFR* tyrosine kinase inhibitors, gefitinib and erlotinib (3, 4), resulting in a paradigm shift in therapeutic strategy, based on *EGFR* mutation status, in these patients (28). Prognosis remains poor, however, in patients with tumors bearing nonmutated *EGFR*. We have shown here that a multiple kinase inhibitor, E7080, suppressed the production of hemato-

genous metastasis into multiple organs and prolonged the survival of mice bearing lung cancer cells with nonmutated *EGFR*. Because E7080 treatment was started after the establishment of micrometastases or macrometastases, our results indicate that E7080 can suppress the enlargement of established metastases. Our results also suggest that the major mechanism by which E7080 inhibited established metastases was inhibition of tumor cell growth due to angiogenesis inhibition. The angiogenesis inhibition was caused by suppression of VEGFR-2 expressed in endothelial cells. Angiogenesis inhibitors indirectly inhibit tumor growth via inhibition of angiogenesis which is essential for supplying the oxygen and nutrition to cancer cells (6). Here, E7080 inhibited the phosphorylation of VEGFR-2 in endothelial cells even at low doses ($IC_{50} = 0.3$ nmol/L) but did not directly inhibit the *in vitro* proliferation of tumor cells at high doses ($IC_{50} > 1,000$ nmol/L). In addition, E7080 dramatically decreased the density of CD31-positive vessels, and decreased the number of proliferating tumor cells, in the metastatic nodules.

We also found that E7080 treatment inhibited lymphangiogenesis. E7080 decreased the number of LYVE-1-positive lymph endothelial cells in the metastatic nodules produced by SBC-5 cells, although this effect was less than its effect on angiogenesis. Although lymphangiogenesis is thought to facilitate lymph node metastasis (7, 10, 11), the impact of lymphangiogenesis on the production of experimental hematogenous

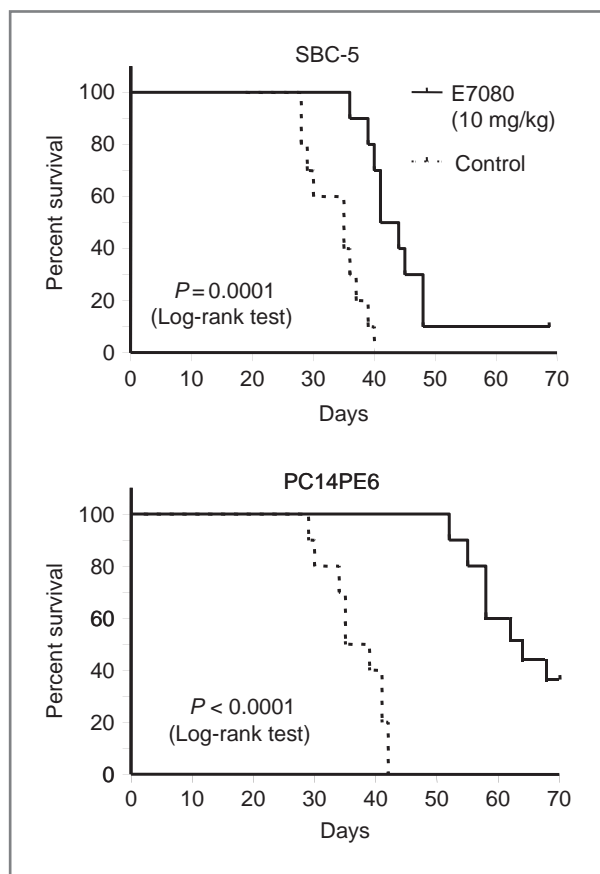


Figure 5. E7080 prolongation of the survival of mice bearing metastases of lung cancer cells bearing nonmutant EGFR. SBC-5 (A) and PC14PE6 (B) cells were intravenously inoculated into NK cell-depleted SCID mice and nude mice, respectively. The mice received distilled water or E7080 (10 mg/kg) orally, starting 14 days after tumor cell inoculation until they became moribund.

metastases in our models remains unclear. Interestingly, SBC-5 cells expressed VEGFR-3 but not its ligands, VEGF-C and VEGF-D. One possible explanation for lymphangiogenesis in SBC-5 tumors is that VEGF-C and/or VEGF-D production by tumor cells was induced *in vivo* (29), or the host microenvironment produced VEGF-C and VEGF-D (30) and activated VEGFR-3 on lymph endothelial cells. Another possibility is that VEGF produced by SBC-5 cells as well as host cells stimulated VEGFR-2, inducing not only angiogenesis but lymphangiogenesis (31). Further investigations on the role of lymphangiogenesis in our models may provide greater understanding of the mechanism by which E7080 inhibited lymphangiogenesis.

Interestingly, H1048 cells did not produce lung metastasis though they originated from lung. This phenomenon was also observed in several SCLC (H69, H69/VP, and SBC-3) and NSCLC (RERF-LC-AI) cells in our metastasis model (20, 32). The reason why these lung cancer cell lines failed to produce lung metastasis in our model is unclear at present. As proposed by Paget as "seed and soil

theory" (33), organ tropism of metastasis may be determined by cross-talk between cancer cells and host microenvironment. We previously reported that SBC-5 cells expressed different sets of genes in the different organs (34), suggesting heterogeneity of lung cancer cells and importance of interaction with microenvironment. We would like to explore the mechanisms in future.

Many chemotherapeutic agents show heterogeneity of therapeutic efficacy among organs (33). One of the advantages of our metastasis models is that therapeutic efficacy can be evaluated in multiple organs simultaneously (22–24). We found that E7080 showed therapeutic effects in almost all organs evaluated. For example, E7080 decreased the total number of osteolytic bone nodules, as well as the number of large nodules (>2 mm diameter) in the liver and kidney. This is consistent with the antiangiogenic mechanism of action of E7080 because angiogenesis is required for enlargement of nodules over 1 to 2 mm in diameter (35). The only exception was that E7080 had little effect on lung nodules produced by SBC-5 cells. However, lung lesions produced by SBC-5 cells were smaller than 2 mm in diameter and had few endothelial cells and lymph endothelial cells. In sharp contrast, PC14PE6 cells, which produce high amounts of VEGF, give rise to highly angiogenic and lymphangiogenic lung lesions greater than 2 mm in diameter (9, 25). E7080 decreased the number of endothelial cells, lymph endothelial cells, and proliferating cells in PC14PE6 lung lesions, but it did not in SBC-5 lung lesions with few endothelial cells and lymph endothelial cells (Supplementary Figs. S4 and 5). In addition, while E7080 did not decrease the total number of lung nodules, it did decrease the number of large nodules, similar to its effect against liver metastases of SBC-5 and H1048 cells (Table 1). Collectively, these results indicate that E7080 possesses antiangiogenic activity in various organs including the lungs.

Another serious problem in the management of patients with lung cancer is pleural effusion, which causes respiratory symptoms and decreases patient quality of life (36). We have reported previously that the VEGF-VEGFR-2 axis plays an essential role in the production of pleural effusion (9). To produce pleural effusion, tumor cells must invade the pleura, disseminate into the pleural cavity, and produce a large amount of VEGF in the thoracic cavity. This VEGF induces hypervascular permeability in the thoracic cavity, thereby causing pleural effusion (9). In addition to suppressing the enlargement of lung nodules by PC14PE6 cells, E7080 may inhibit vascular permeability by inhibiting VEGFR-2 activity. Therefore, E7080 may be also useful for controlling pleural effusion caused by lung cancers producing high amounts of VEGF.

We previously reported that 2 other VEGFR-2 tyrosine kinase inhibitors, PTK787 (37) and ZD6474 (vandetanib; refs. 25, 38), have therapeutic efficacy against multiple organ metastases induced by SBC-5 cells and/or against pleural effusion induced by PC14PE6 cells. The results

presented here indicate that E7080 is much more active than these other compounds. The concentrations of E7080 required to inhibit endothelial proliferation was lower, as was the dose needed to show antimetastatic effects (10 vs. 50 mg/kg; refs. 25, 37, 38). Furthermore, we found that E7080 prolonged survival in mice bearing multiple organ metastases of SBC-5 cells those with pleural effusion caused by PC14PE6 cells, clearly indicating that E7080 is a highly active inhibitor of angiogenesis of lung cancers with nonmutant EGFR. Short-term (2 weeks) treatment with E7080 did prolong the mice bearing multiple organ metastases of SBC-5 or PC14PE6 cells, the duration was only 1 to 2 weeks (data not shown). Therefore, continuous treatment may be necessary for obtaining best therapeutic efficacy of this compound.

E7080 is currently being evaluated in clinical trials against a wide variety of solid tumors. Phase I studies have shown that treatment with E7080 induced partial responses in patients with colorectal cancer, melanoma, renal cell carcinoma, and sarcoma (39, 40). Based on these promising results, phase II clinical trials are currently ongoing.

In summary, we found that the multi tyrosine kinase inhibitor E7080 could inhibit angiogenesis and the enlargement of hematogenic metastatic nodules, thereby

prolonging survival of mice bearing multiple organ metastases and pleural effusion induced by lung cancer cells with nonmutant EGFR. Further clinical evaluation of E7080 is warranted for treatment of lung cancer patients with nonmutant EGFR.

Disclosure of Potential Conflicts of Interest

S. Sone received research support and lecture fee from Eisai Co. Ltd. The other authors declare no potential conflicts of interest. A. Tsuruoka and T. Uenaka are employed by Eisai Co. Ltd.

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References

- Gralow J, Ozols RF, Bajorin DF, Cheson BD, Sandler HM, Winer EP, et al. American Society of Clinical Oncology. Clinical cancer advances 2007: major research advances in cancer treatment, prevention, and screening—a report from the American Society of Clinical Oncology. *J Clin Oncol* 2008;26:313–25.
- Johnson DH, Blot WJ, Carbone DP, Gonzalez A, Hallahan D, Massion PP, et al. Cancer of the lung: non-small cell lung cancer and small cell lung cancer. In: *Abeloff's clinical oncology*. 4th ed. Philadelphia: Churchill Livingstone Elsevier; 2008. p. 1307–66.
- Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–8.
- Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–8.
- Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–57.
- Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005;438:967–74.
- Lohela M, Bry M, Tammela T, Alitalo K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr Opin Cell Biol* 2009;21:154–65.
- Luo JC, Yamaguchi S, Shinkai A, Shitara K, Shibuya M. Significant expression of vascular endothelial growth factor/vascular permeability factor in mouse ascites tumors. *Cancer Res* 1998;58:2652–60.
- Yano S, Shinohara H, Herbst RS, Kuniyasu H, Bucana CD, Ellis LM, et al. Production of experimental malignant pleural effusions is dependent on invasion of the pleura and expression of vascular endothelial growth factor/vascular permeability factor by human lung cancer cells. *Am J Pathol* 2000;157:1893–903.
- Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 1997;16:3898–911.
- Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001;7:192–8.
- Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;355:2542–50.
- Reck M, von Pawel J, Zatloukal P, Ramlau R, Gorbounova V, Hirsh V, et al. Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAIL. *J Clin Oncol* 2009;27:1227–34.
- Reck M, von Pawel J, Zatloukal P, Ramlau R, Gorbounova V, Hirsh V, et al. Overall survival with cisplatin-gemcitabine and bevacizumab or placebo as first-line therapy for nonsquamous non-small-cell lung cancer: results from a randomised phase III trial (AVAIL). *Ann Oncol* 2010;21:1804–9.
- Matsui J, Yamamoto Y, Funahashi Y, Tsuruoka A, Watanabe T, Wakabayashi T, et al. E7080, a novel inhibitor that targets multiple kinases, has potent antitumor activities against stem cell factor producing human small cell lung cancer H146, based on angiogenesis inhibition. *Int J Cancer* 2008;122:664–71.
- Matsui J, Funahashi Y, Uenaka T, Watanabe T, Tsuruoka A, Asada M. 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. *Clin Cancer Res* 2008;14:5459–65.
- Ikuta K, Yano S, Trung VT, Hanibuchi M, Goto H, Li Q, et al. E7080, a multi-tyrosine kinase inhibitor, suppresses the progression of malignant pleural mesothelioma with different proangiogenic cytokine production profiles. *Clin Cancer Res* 2009;15:7229–37.
- Kiura K, Watarai S, Shibayama T, Ohnishi T, Kimura I, Yasuda T. Inhibitory effects of cholera toxin on *in vitro* growth of human lung cancer cell lines. *Anticancer Drug Des* 1993;8:417–28.
- Yano S, Wang W, Li Q, Sakurama H, Nakamura T, Ogino H, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with EGF receptor mutations. *Cancer Res* 2008;68:9479–87.

20. Yano S, Nishioka Y, Izumi K, Tsuruo T, Tanaka T, Miyasaka M, et al. Novel metastasis model of human lung cancer in SCID mice depleted of NK cells. *Int J Cancer* 1996;67:211–7.
21. Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* 1984;70:257–68.
22. Miki T, Yano S, Hanibuchi M, Sone S. Bone metastasis model with multiorgan dissemination of human small-cell lung cancer (SBC-5) cells in natural killer cell-depleted SCID mice. *Oncol Res* 2000;12:209–17.
23. Miki T, Yano S, Hanibuchi M, Kanematsu T, Maguruma H, Sone S. Parathyroid hormone-related protein (PTHrP) is responsible for production of bone metastasis, but not visceral metastasis, by human small cell lung cancer SBC-5 cells in natural killer cell-depleted SCID mice. *Int J Cancer* 2004;108:511–5.
24. Muguruma H, Yano S, Kakiuchi S, Uehara H, Kawatani M, Osada H, et al. Reveromycin A inhibits osteolytic bone metastasis of small-cell lung cancer cells, SBC-5, through an anti-osteoclastic activity. *Clin Cancer Res* 2005;11:8822–8.
25. Matsumori Y, Yano S, Goto H, Nakataki E, Wedge SR, Ryan AJ, et al. ZD6474, an inhibitor of vascular endothelial growth factor receptor tyrosine kinase, inhibits growth of experimental lung metastasis and production of malignant pleural effusions in a non-small cell lung cancer model. *Oncol Res* 2006;16:15–26.
26. Sauter B, Foedinger D, Sterniczky B, Wolff K, Rappersberger K. Immunoelectron microscopic characterization of human dermal lymphatic microvascular endothelial cells. Differential expression of CD31, CD34, and type IV collagen with lymphatic endothelial cells vs blood capillary endothelial cells in normal human skin, lymphangioma, and hemangioma in situ. *J Histochem Cytochem* 1998;46:165–76.
27. Whitehurst B, Flister MJ, Bagaikar J, Volk L, Bivens CM, Pickett B, et al. Anti-VEGF-A therapy reduces lymphatic vessel density and expression of VEGFR-3 in an orthotopic breast tumor model. *Int J Cancer* 2007;121:2181–91.
28. Herbst RS, Heymach JV, Lippman SM. Lung cancer. *N Engl J Med* 2008;359:1367–80.
29. Nakamura Y, Yasuoka H, Tsujimoto M, Yoshidome K, Nakahara M, Nakao K, et al. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. *Clin Cancer Res* 2006;12:1201–7.
30. Granata F, Frattini A, Loffredo S, Staiano RI, Petraroli A, Ribatti D, et al. Production of vascular endothelial growth factors from human lung macrophages induced by group IIA and group X secreted phospholipases A2. *J Immunol* 2010;184:5232–41.
31. Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 2004;113:1040–50.
32. Hanibuchi M, Yano S, Nishioka Y, Yanagawa H, Kawano T, Sone S. Therapeutic efficacy of mouse-human chimeric anti-ganglioside GM2 monoclonal antibody against multiple organ micrometastases of human lung cancer in NK cell-depleted SCID mice. *Int J Cancer* 1998;78:480–5.
33. Paget S. Distribution of secondary growths in cancer of the breast. *Lancet* 1889;1:571.
34. Kakiuchi S, Daigo Y, Tsunoda T, Yano S, Sone S, Nakamura Y. Genome-wide analysis of organ-preferential metastasis of human small cell lung cancer in mice. *Mol Cancer Res* 2003;1:485–99.
35. Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 1994;79:185–8.
36. Light RW. Pleural effusions related to metastatic malignancies. In: Light RW, editor. *Pleural diseases*. 4th ed. Philadelphia, PA: Wilkins; 2001. p. 108–34.
37. Yano S, Herbst RS, Shinohara H, Knighton B, Bucana CD, Killion JJ, et al. Treatment for malignant pleural effusion of human lung adenocarcinoma by inhibition of vascular endothelial growth factor receptor tyrosine kinase phosphorylation. *Clin Cancer Res* 2000;6:957–65.
38. Yano S, Muguruma H, Matsumori Y, Goto H, Nakataki E, Edakuni N, et al. Antitumor vascular strategy for controlling experimental metastatic spread of human small cell lung cancer cells with ZD6474 in natural killer cell-depleted severe combined immunodeficiency mice. *Clin Cancer Res* 2005;11:8789–98.
39. Yamada K, Yamamoto N, Yamada Y, Nokihara H, Fujiwara Y, Hirata T, et al. Phase I dose escalation study and biomarker analysis of E7080 in patients with advanced solid tumors. *Clin Cancer Res* 2011;17:2528–37.
40. Glen H, Boss D, Morrison R, Roelvink M, Wanders J, Mazur A, et al. A phase I dose escalation and pharmacokinetic study of E7080, a small molecule tyrosine kinase inhibitor, in patients with advanced malignancies. In: 2008 ASCO Annual Meeting; 2008. Abstract 3526 handout.