

Lenvatinib in combination with golvatinib overcomes hepatocyte growth factor pathway-induced resistance to vascular endothelial growth factor receptor inhibitor

Takayuki Nakagawa,¹ Tomohiro Matsushima,¹ Satoshi Kawano,¹ Youya Nakazawa,¹ Yu Kato,¹ Yusuke Adachi,¹ Takanori Abe,¹ Taro Semba,² Akira Yokoi,¹ Junji Matsui,¹ Akihiko Tsuruoka² and Yasuhiro Funahashi^{1,3}

¹Tsukuba Research Laboratory, Eisai Co., Ltd., Tsukuba; ²Eisai Co., Ltd., Tokyo, Japan; ³Eisai Inc., Andover, Massachusetts, USA

Key words

Angiogenesis, hepatocyte growth factor signaling, Met, resistance, vascular endothelial growth factor receptor

Correspondence

Yasuhiro Funahashi, Eisai Inc., 4 Corporate Drive, Andover, Massachusetts 01810, USA.
Tel: +1-978-837-4807; Fax: +1-978-689-0543;
E-mail: yasuhiro_funahashi@eisai.com

Funding Information

Eisai Co., Ltd.

Received December 16, 2013; Revised March 26, 2014;
Accepted March 31, 2014

Cancer Sci 105 (2014) 723–730

doi: 10.1111/cas.12409

Vascular endothelial growth factor receptor (VEGFR) inhibitors are approved for the treatment of several tumor types; however, some tumors show intrinsic resistance to VEGFR inhibitors, and some patients develop acquired resistance to these inhibitors. Therefore, a strategy to overcome VEGFR inhibitor resistance is urgently required. Recent reports suggest that activation of the hepatocyte growth factor (HGF) pathway through its cognate receptor, Met, contributes to VEGFR inhibitor resistance. Here, we explored the effect of the HGF/Met signaling pathway and its inhibitors on resistance to lenvatinib, a VEGFR inhibitor. In *in vitro* experiments, addition of VEGF plus HGF enhanced cell growth and tube formation of HUVECs when compared with stimulation by either factor alone. Lenvatinib potently inhibited the growth of HUVECs induced by VEGF alone, but cells induced by VEGF plus HGF showed lenvatinib resistance. This HGF-induced resistance was cancelled when the Met inhibitor, golvatinib, was added with lenvatinib. Conditioned medium from tumor cells producing high amounts of HGF also conferred resistance to inhibition by lenvatinib. In s.c. xenograft models based on various tumor cell lines with high HGF expression, treatment with lenvatinib alone showed weak antitumor effects, but treatment with lenvatinib plus golvatinib showed synergistic antitumor effects, accompanied by decreased tumor vessel density. These results suggest that HGF from tumor cells confers resistance to tumor endothelial cells against VEGFR inhibitors, and that combination therapy using VEGFR inhibitors with Met inhibitors may be effective for overcoming resistance to VEGFR inhibitors. Further evaluation in clinical trials is warranted.

Angiogenesis, the formation of new networks of blood vessels from existing vessels, contributes to the progression of cancer, including tumor growth and metastasis. Vascular endothelial growth factor plays a major role in angiogenesis.^(1,2) A number of inhibitors that target vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs), including bevacizumab, sunitinib, sorafenib, and pazopanib, have been approved for use in the treatment of certain types of cancers, and numerous other inhibitors that target the VEGF signaling pathway are in clinical development.^(1,2) Although inhibitors that target VEGF or VEGFRs have been shown to inhibit tumor angiogenesis and suppress tumor growth when they are used either as single agents or in combination with chemotherapy, their therapeutic efficacy has not been indicated for all cancer types.^(3,4) These inhibitors are ineffective against subgroups of tumors that display intrinsic resistance, even in those cancer types for which they have been approved, and some patients who initially respond to treatment acquire resistance over time. Previous research has shown that the following

factors are involved in resistance to VEGF inhibitors: expression of fibroblast growth factor-2 and placental growth factor; activation of the Notch signaling pathway through delta-like 4 upregulation of Bv8 (*Bombina variegata* 8 kDa; prokineticin 2) in CD11b⁺Gr1⁺ myeloid cells in a tumor microenvironment; and expression of platelet derived growth factor-C in tumor-associated fibroblasts.^(5–10) The elucidation of other mechanisms behind resistance to VEGF pathway inhibitors is required.

Hepatocyte growth factor is a 90-kDa secretory protein that activates intracellular signal transduction through various pathways (e.g. Ras/Mek/Erk, PI3K/Akt, and Stat3), through its sole receptor, Met receptor tyrosine kinase, to enhance angiogenesis and the capacity of cells to proliferate, survive, and migrate.⁽¹¹⁾ The hepatocyte growth factor (HGF) pathway contributes to the malignant transformation of cancer and is a focus of molecular targeted therapies.^(12,13) Although HGF in tumors is mainly produced by fibroblasts and other tumor interstitial cells, it is also expressed by cancer cells themselves.

Overexpression of HGF occurs in a variety of tumor types and is a poor prognostic factor for some tumor types.⁽¹²⁾ Met is expressed in epithelial cells, as well as endothelial cells, neural cells, hematopoietic cells, and pericytes. Like HGF overexpression, overexpression of Met is associated with poor prognosis in many cancer types.⁽¹²⁾ Furthermore, the HGF/Met signaling pathway has been implicated in resistance to molecular targeted drugs for various types of cancer, including epidermal growth factor receptor inhibitors for epidermal growth factor receptor mutant non-small-cell lung cancer, anaplastic lymphoma kinase inhibitors for anaplastic lymphoma kinase fusion-positive non-small-cell lung cancer, and BRAF inhibitors for *BRAF* V600E mutation-positive melanoma.^(14–17) In a clinical trial of a VEGFR inhibitor, sorafenib, for the treatment of hepatocellular carcinoma, progression-free survival was significantly shorter in patients with high serum HGF levels relative to those with low serum HGF levels.⁽¹⁸⁾ Furthermore, HGF levels rose before tumors re-enlarged during the treatment of metastatic colorectal cancer with regimens including another VEGFR inhibitor, bevacizumab, suggesting an association between the HGF pathway and resistance to VEGFR inhibitors.⁽¹⁹⁾

Lenvatinib is a low molecular weight, orally available inhibitor of VEGFR, and clinical trials for its use in the treatment of several types of cancer are currently underway.⁽²⁰⁾ Here, we showed that the HGF pathway is involved in resistance to lenvatinib treatment, and that combined use of lenvatinib and golvatinib, a low molecular weight, orally available inhibitor of Met,⁽²¹⁾ is effective in overcoming this resistance in a preclinical model.

Materials and Methods

Compounds and reagents. Lenvatinib mesylate (E7080 mesylate; 4-[3-chloro-4-(*N'*-cyclopropylureido)phenoxy]-7-methoxyquinoline-6-carboxamide methanesulfonate mesylate), and golvatinib tartrate (E7050 tartrate; N-[2-fluoro-4-({2-[4-(4-methylpiperazin-1-yl)piperidin-1-yl]carbonylamino}pyridin-4-yl)oxy)phenyl]-*N'*-(4-fluorophenyl)cyclopropane-1,1-dicarboxamide (2*R*,3*R*)-tartrate) were synthesized at Eisai Co., Ltd (Tsukuba, Japan) (Fig. 1a). Recombinant human HGF, recombinant human VEGF, and anti-human HGF neutralizing antibody were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against phospho-Met (Y1234/Y1235) (D26), phospho-VEGFR2 (Y996), phospho-Akt (S473), phospho-Erk1/2 (T202/Y204), Erk1/2 (Cell Signaling Technology, Beverly, MA, USA), Met (C-28), VEGFR2 (C-1158), and Akt (H-136) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GAPDH (Sigma, St. Louis, MO, USA) were used for Western blot analyses.

In vitro kinase assay. For *in vitro* receptor kinase assays, compound or vehicle was mixed in a 96-well round plate with 15 ng enzyme, 250 ng poly-GT-biotin substrate (CIS Biointernational, Ceze Cedex, France), 1 μ M ATP disodium (Sigma), and 0.1% (w/v) BSA in kinase buffer. After incubation for 10 min at 30°C, the reaction was stopped by addition of 0.5 M EDTA. The amount of phosphorylated substrate was determined by means of homogeneous time-resolved fluorescence by using the Discovery HTRF microplate analyzer BD10011 (Packard, Meriden, CT, USA).

Cell culture. Human umbilical vein endothelial cells were isolated as described previously⁽²²⁾ and cultured on type I

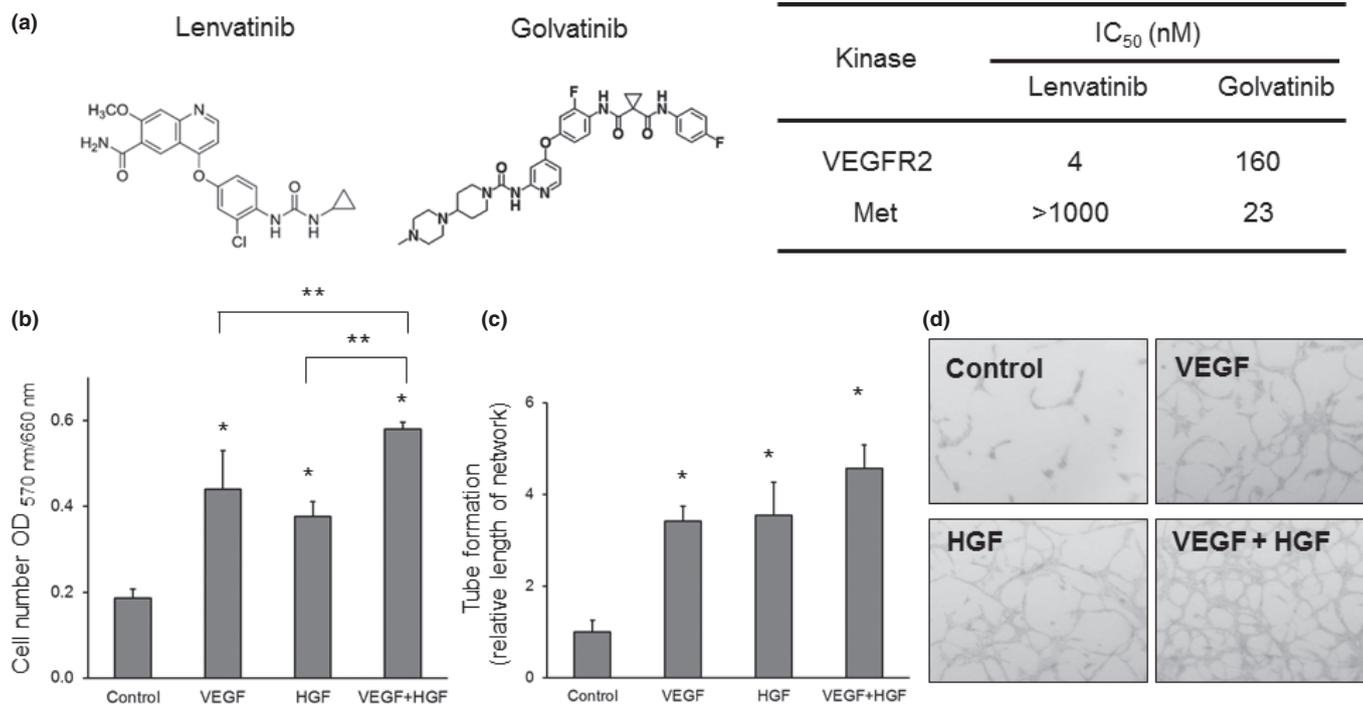


Fig. 1. Effects of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) on cell proliferation and tube formation of endothelial cells *in vitro*. (a) Chemical structure of lenvatinib (left panel), golvatinib (middle panel), and the inhibitory activity of compounds against vascular endothelial growth factor receptor 2 (VEGFR2) and Met (right panel). (b) HUVECs were incubated for 3 days in the presence of VEGF (20 ng/mL), HGF (30 ng/mL), or both. Cell numbers were quantified using the sulforhodamine B method. Data represent means \pm SD. * P < 0.05 versus control; ** P < 0.05 versus the indicated group. (c) Quantification of HUVEC tube formation induced with VEGF, HGF, or both. The relative length of network was calculated relative to the control. Data represent means \pm SD. * P < 0.05 versus control. (d) Representative images of tube formation induced by VEGF, HGF, or both.

collagen-coated plates at 37°C with 5% CO₂ in EGM-2 Bullet kit medium (Eidia, Tokyo, Japan). Cells from passages 3 to 6 were used in experiments. The human melanoma cell line SEKI was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and the human gastric cancer cell line IM95m was obtained from the Health Science Research Resources Bank (Osaka, Japan). The human pancreatic cancer cell line KP-4 was a gift from Dr. Akihiko Funakoshi at the National Kyushu Cancer Center (Fukuoka, Japan). The human ovarian cancer cell line A2780 was obtained from the ATCC (Manassas, VA, USA). Tumor cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS with the exception of IM95m cells, which were maintained in DMEM containing 4500 mg/L glucose, 10 µg/mL insulin, and 10% (v/v) FBS. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Cell proliferation assay. A cell proliferation assay was carried out by modification of the sulforhodamine B colorimetric assay.⁽²³⁾ The HUVECs were plated at a density of 2.0×10^3 cells/well in a microtiter plate. After overnight incubation, serial dilutions of DMSO (control) or compound were added and the cells were further incubated in the presence of VEGF (20 ng/mL), or HGF (30 ng/mL), or both. Neither VEGF nor HGF was added to the control. After cell culture for 3 days, the medium was discarded and the cells were fixed with 100 µL of 10% (v/v) trichloroacetic acid solution (Wako Pure Chemical Industries, Osaka, Japan). After extensive washing, the cells were stained with sulforhodamine B dye dissolved in Tris-based solution (pH 10.5). The absorbance of each well was measured with an MTP-500 microplate reader (Corona Electric, Hitachinaka, Japan) at 570 nm (reference wavelength, 660 nm).

Sandwich tube formation assay. Four-hundred microliter aliquots of collagen gel was added to 24-well plates and allowed to gel for at least 1 h at 37°C. The HUVECs were then plated on the gel at a concentration of 1.2×10^5 cells/well in human endothelial-SFM basal growth medium (Life Technologies, Grand Island, NY, USA) with 10 ng/mL epidermal growth factor (Life Technologies) and either 30 ng/mL HGF (Life Technologies) or 20 ng/mL VEGF (Wako Pure Chemical Industries). After culture at 37°C overnight, HUVECs were covered with another 400 µL collagen gel, which was allowed to gel for at least 3 h at 37°C. Then human endothelial-SFM basal growth medium (1.5 mL) supplemented with epidermal growth factor and HGF, VEGF, or both were added. The tube length of capillaries was quantified by tracing the center of each formed tube and calculating the pixel intensity of the outlined image using the Mac SCOPE 2.56 software (Mitani, Chiba, Japan).

Assay for growth factor expression levels. Tumor cells (1×10^5) were cultured in RPMI-1640 medium with 10% (v/v) FBS for 24 h, washed with PBS, and incubated for an additional 24 h in 1 mL RPMI-1640 medium with 1% (v/v) FBS in a 24-well culture plate. The culture medium was harvested and centrifuged, and the supernatant was stored at -80°C until analysis. Growth factor concentrations were determined by comparison to a standard curve using Quantikine ELISA kits (R&D Systems), as recommended by the manufacturer. All samples were assayed in triplicate.

Animal studies. Nude mice (CAnN.Cg-Foxn1nu/CrlCrlj; 5–6-week-old females) were obtained from Charles River Laboratories Japan (Kanagawa, Japan). Mice were maintained under specific pathogen-free conditions and housed in barrier facilities on a 12:12-h light:dark cycle, with food and water *ad libitum*.

Cultured tumor cells (5×10^6 cells/mice) were implanted s.c. into the flanks of the mice. When the tumor volume reached 100–300 mm³, the mice were randomized into groups. Lenvatinib and golvatinib were dissolved in sterile distilled water and given orally once a day for 8–21 days. Mice were killed 24 h after the final treatment. Following treatment, each tumor was measured in two dimensions, and the volume was calculated by using the following formula: tumor volume (mm³) = $1/2 \times \text{length (mm)} \times [\text{width (mm)}]^2$. All animal experiments were carried out in accordance with the guidelines for animal experiments of Eisai Co., Ltd. Differences in relative tumor volume between vehicle-treated and compound-treated groups were analyzed using one-way ANOVA followed by Dunnett's multiple-comparison test. Combination effects were analyzed using two-way ANOVA. *P*-values of <0.05 (two-sided) were considered statistically significant.

Immunohistochemistry. Tumors were collected and fixed with zinc fixative or formalin and paraffin-embedded blocks were prepared. Sections were stained as previously described.⁽²¹⁾ All histological specimens were viewed with a CCD Hyper Scope (Keyence, Osaka, Japan). Microvessel density was determined as described previously.⁽²⁴⁾ Briefly, four or five fields per tumor were scored for tumor microvessels at 400× magnification, and tumor microvessel densities (i.e. number of microvessel/field) were calculated.

Statistical analysis. Data are expressed as the mean ± SD. Differences between experimental groups were analyzed using Student's *t*-test. *P*-values of <0.05 were considered statistically significant. Statistical analyses were carried out using the SAS 8.1 software package (SAS Institute Japan, Tokyo, Japan).

Results

Hepatocyte growth factor cooperates with VEGF to promote angiogenesis and confer resistance to proliferation-inhibitory effect of lenvatinib in HUVECs. We first examined the effects of VEGF and HGF in HUVECs, an *in vitro* angiogenesis model. Addition of either VEGF or HGF significantly promoted cell proliferation and network formation of capillary-like vessels of HUVECs compared to that observed with vehicle alone (control) (Fig. 1b,c). Costimulation with VEGF and HGF enhanced proliferation and tube formation of HUVECs to a significantly greater extent than that obtained with either single treatment (Fig. 1b,c), and the networks formed after costimulation were more dense and intricately branched than those induced by either single factor (Fig. 1d). We next examined the proliferation-inhibiting activity of lenvatinib, which is a potent VEGFR inhibitor, but not a Met inhibitor (Fig. 1a). In HUVECs stimulated with VEGF alone, lenvatinib inhibited proliferation at IC₅₀ 1.6 nM and reached a plateau of approximately 80% inhibition (Fig. 2a). In comparison, treatment with VEGF plus HGF showed decreased inhibitory activity of lenvatinib (IC₅₀, 5.5 nM; plateau of approximately 60% inhibition), indicating that the HUVECs to which HGF had been added were resistant to lenvatinib.

We then tested the effect of golvatinib, an ATP-competitive Met inhibitor (Fig. 1a) on HGF-induced resistance to lenvatinib (Fig. 2b). When HUVECs stimulated with VEGF plus HGF were treated with golvatinib in combination with lenvatinib, the proliferation was potently inhibited to a significantly greater extent than that observed when either golvatinib or lenvatinib were used alone (Fig. 2b). To clarify the signal transduction events in endothelial cells, HUVECs were treated with VEGF, HGF, or both HGF and VEGF. Vascular

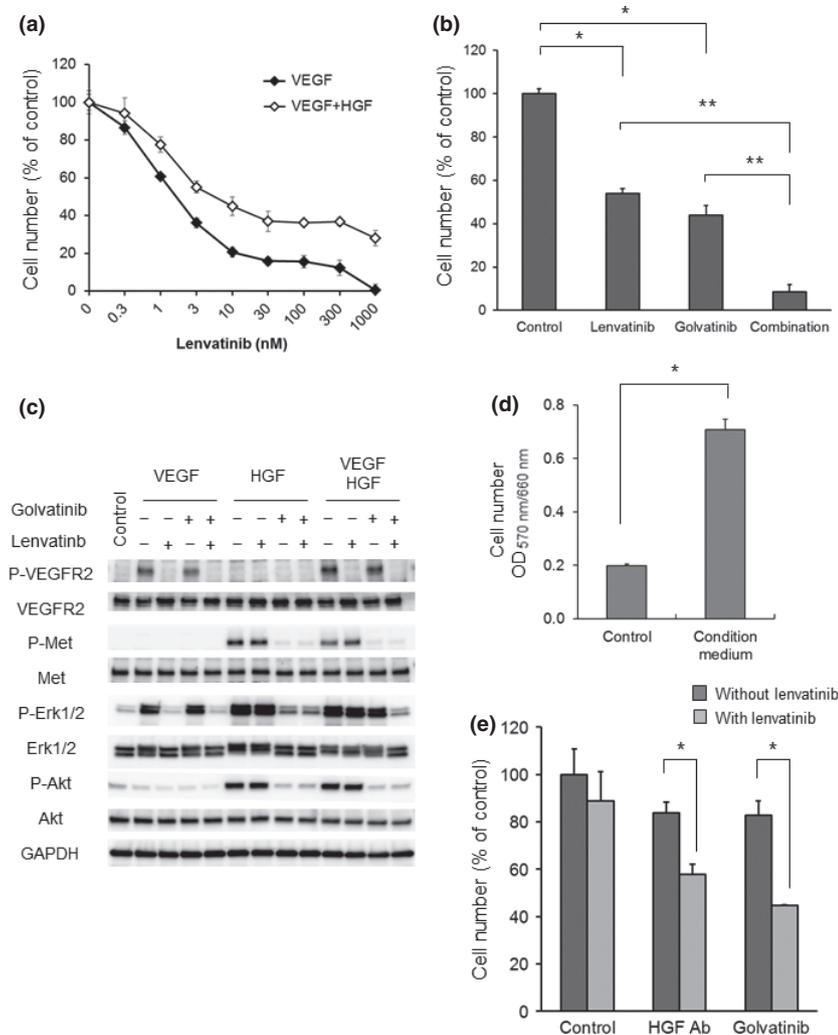


Fig. 2. Effect of combined hepatocyte growth factor (HGF)/Met signaling pathway inhibitors on HGF-induced resistance to lenvatinib inhibition of HUVEC proliferation *in vitro*. (a) HUVECs were incubated with various concentrations of lenvatinib in the presence of vascular endothelial growth factor (VEGF) or VEGF plus HGF. (b) HUVECs were incubated with VEGF plus HGF for 3 days, then treated with lenvatinib (5 nM), golvatinib (30 nM), or both. Data represent means \pm SD. * P < 0.05 versus control; ** P < 0.05 versus the indicated group. (c) HUVECs were treated with DMSO control, lenvatinib (10 nM), golvatinib (50 nM), or both lenvatinib and golvatinib for 1 h. Additionally, cells were stimulated with medium, VEGF (20 ng/mL), HGF (30 ng/mL), or both VEGF and HGF for 10 min. The cell lysates were then harvested and the indicated proteins were analyzed by Western blotting. (d) HUVECs were incubated with or without (control) conditioned medium from SEKI human melanoma cells for 3 days. (e) HUVECs were incubated with conditioned medium from SEKI cells for 3 days, then treated with lenvatinib (10 nM) alone or in combination with either human HGF neutralizing Ab (10 μ g/mL) or golvatinib (50 nM). Data represent means \pm SD. * P < 0.05 versus the indicated group.

endothelial growth factor induced phosphorylation of VEGFR2 and Erk1/2, which was inhibited by lenvatinib. Hepatocyte growth factor induced phosphorylation of Met, Akt, and Erk1/2, which was inhibited by golvatinib, but not lenvatinib (Fig. 2c). When HUVECs were treated with VEGF and HGF, lenvatinib inhibited the phosphorylation of VEGFR2, but Akt and Erk1/2 remained activated. The phosphorylation of Akt and Erk1/2 was inhibited when lenvatinib was combined with the Met inhibitor golvatinib (Fig. 2c). These results suggest HGF confers resistance to VEGF-driven HUVECs against lenvatinib, and that combined treatment with golvatinib circumvents this resistance.

SEKI is a human melanoma cell line, and its culture supernatant is rich in both HGF and VEGF (Table 1). SEKI culture supernatant significantly enhanced proliferation of HUVECs compared with the control (Fig. 2d). Single treatment with either lenvatinib, human HGF-neutralizing antibody, or golvatinib had moderate inhibitory effect on the proliferation of HUVECs promoted by SEKI culture supernatant (Fig. 2e). In contrast, the combined use of lenvatinib with either human HGF-neutralizing antibody or golvatinib resulted in significant inhibition of the proliferation of HUVECs promoted by SEKI culture supernatant (Fig. 2e). These results suggest that VEGF and HGF produced from SEKI cells promoted the proliferation of HUVECs, and that HGF in the SEKI culture supernatant conferred resistance to lenvatinib treatment.

Combination treatment of lenvatinib and golvatinib has a strong antitumor effect in HGF-overexpressing human tumor xenografts in nude mice. SEKI, KP-4, IM95m, and A2780 human cancer cell lines, which are derived from melanoma, gastric cancer, pancreatic cancer, and ovarian cancer, respectively, are known to produce both HGF and VEGF.^(25–27) We confirmed that each cell line expressed similar or higher amounts of HGF compared to VEGF *in vitro*, with the amount of HGF secreted by SEKI and A2780 cells being seven to eight times the amount of VEGF (Table 1). The expression of placental growth factor, fibroblast growth factor-2, and platelet derived growth factor-C, which are growth factors reported to contribute to VEGF resistance, was undetectable or low compared with the HGF expression level in these cell lines (Table 1). Lenvatinib showed weak or no inhibitory activity (IC_{50} , >1 μ M) against the proliferation of these HGF-producing cell lines (Table 1). Lenvatinib also did not affect the production of VEGF or HGF from cancer cells (Fig. S1a,b). Golvatinib exhibited inhibitory activity against the IM95m cell line (IC_{50} , 27 nM), but IC_{50} values for the other three cell lines were all >1 μ M. SEKI and A2780 did not show obvious expression of Met. In contrast, Met expression and activation was observed in KP-4 and IM95m (Fig. S2). Treatment with golvatinib inhibited phosphorylation of Met in KP-4 and IM95m, partially inhibited phosphorylation of Akt in KP-4, and partially inhibited phosphorylation of Erk1/2 in IM95m.

Table 1. Growth factor production and growth inhibitory activity of compounds in tumor cell lines

Name	SEKI	KP-4	IM95m	A2780
Tumor type	Melanoma	Pancreatic tumor	Gastric tumor	Ovarian tumor
Growth factor production (pg/1 × 10 ⁵ cells/24 h)				
VEGF	300.8 ± 22.6	220.6 ± 3.5	128.1 ± 5.8	492.3 ± 41.2
HGF	21 152.4 ± 1434.5	207.0 ± 81.1	2831.0 ± 44.3	33 310.3 ± 2837.0
PIGF	74.5 ± 1.8	BLQ	BLQ	BLQ
FGF-2	BLQ	BLQ	BLQ	BLQ
PDGF-C	BLQ	118.0 ± 5.9	BLQ	BLQ
Growth inhibitory activity IC ₅₀ (μM)				
Lenvatinib	>1	>1	>1	>1
Golvtatinib	>1	>1	0.027	>1

BLQ, below the limit of quantitation; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; PDGF-C, platelet derived growth factor-C; PIGF, placental growth factor; VEGF, vascular endothelial growth factor.

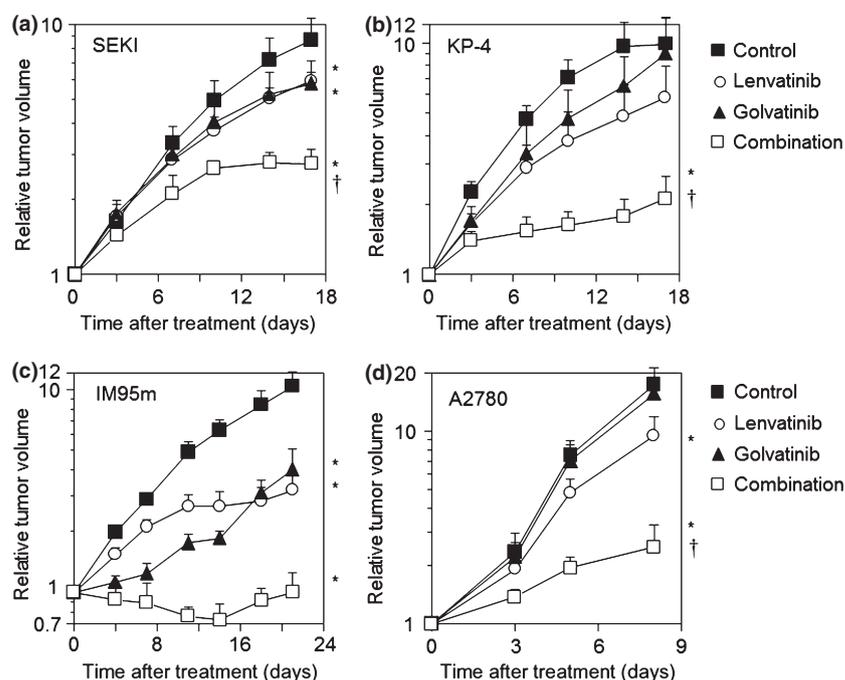


Fig. 3. Effect of combined lenvatinib and golvtatinib treatment on tumor volume in hepatocyte growth factor (HGF)-expressing human cancer xenograft models in mice. Nude mice bearing SEKI (a), KP-4 (b), IM95m (c), or A2780 (d) tumors were treated orally with lenvatinib (10 mg/kg), golvtatinib (100 mg/kg), or both, once daily. Tumor volume was measured using calipers on the indicated days. Data represent the means ± SD of six animals. * $P < 0.05$ versus vehicle control at the end of treatment. †Combination therapy demonstrated a synergistic effect (two-way ANOVA analysis; $P < 0.05$).

Neither the Akt nor the Erk1/2 phosphorylation level changed in SEKI or A2780 when treated with golvtatinib (Fig. S2). Treatment with golvtatinib slightly decreased the expression of VEGF in KP-4 and IM95m cells, but did not affect the VEGF expression of SEKI or A2780 (Fig. S1a,b).

We used the above cell lines to create mouse human tumor xenograft models to test the therapeutic effects of lenvatinib and golvtatinib given alone or in combination (Fig. 3). A clinically relevant dose of lenvatinib (10 mg/kg) showed significant but weak antitumor activity in all but the KP-4 model (Fig. 3). Administration of a clinically relevant dose of golvtatinib (100 mg/kg) showed similar antitumor activity to lenvatinib in the SEKI and IM95m models, but showed no antitumor activity in the KP-4 and A2780 models. In contrast, combined treatment with lenvatinib and golvtatinib showed significant antitumor activities in all four models examined when compared with the control, and in all but the IM95m model when compared with treatment using each agent alone. The *in vivo* antitumor activities were synergistic in the SEKI, KP-4, and A2780 models, and additive in the IM95m model. Neither abnormal macroscopic findings nor body weight loss were

evident in mice treated with either lenvatinib or golvtatinib alone or in combination (Fig. S3).

Combination treatment with lenvatinib and golvtatinib decreases tumor microvessel density in xenografted tumors in nude mice. The surfaces of the A2780 xenograft tumors were reddish, but became pale with combination treatment (Fig. 4a). We then tested whether the antitumor effects of combined treatment with lenvatinib and golvtatinib were associated with inhibition of tumor angiogenesis in human tumor xenograft models. Consistent with our macroscopic observations, immunohistochemical staining of A2780 xenograft tumors, which were excised from mice treated with compound for 7 days, with antibodies against the endothelial cell marker CD31 demonstrated a decrease in endothelial cell staining with combination treatment (Fig. 4b,c) compared with the control or with each treatment individually. Combination treatment also decreased cancer cell proliferation and induced apoptosis of cancer cells (Figs 4d,e,S4a,b). Combination treatment also resulted in a significant decrease in tumor microvessel density in the SEKI and KP-4 models, which were treated with compound for 4 days (Fig. S4c,d). Consistent with our *in vitro*

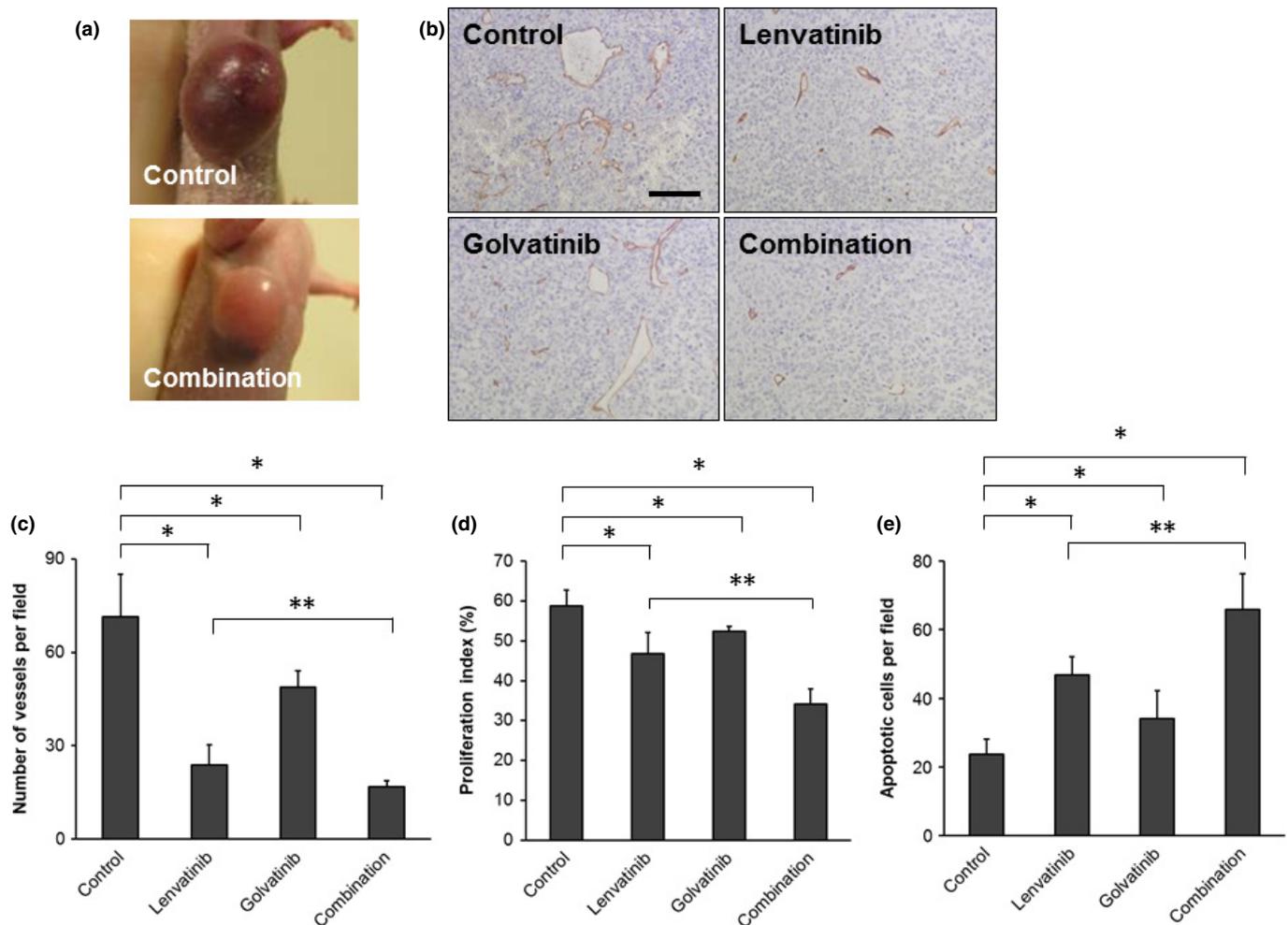


Fig. 4. Effect of combination lenvatinib and golvatinib treatment on anti-angiogenic activity in hepatocyte growth factor-expressing human cancer xenograft models in nude mice. (a) Macroscopic photographs of an A2780 s.c. tumor treated with vehicle (control) or combined treatment with lenvatinib and golvatinib. (b) Representative image of CD31 immunohistochemical staining of tumor microvessels in A2780 xenografted tumors in nude mice treated with lenvatinib, golvatinib, or both for 7 days. Bar = 100 μ m. (c–e) Quantitative analysis of tumor microvessel density with CD31 staining (c), proliferation index with Ki67 staining (d), and apoptotic cells with TUNEL staining (e) in A2780 xenografted tumors treated with lenvatinib, golvatinib, or both for 7 days. Data represent means \pm SD. * P < 0.05 versus control; ** P < 0.05 versus the indicated group.

findings, these results suggest that the simultaneous inhibition of VEGFR with lenvatinib and inhibition of Met with golvatinib reduced angiogenesis in the HGF- and VEGF-overexpressing xenograft tumors, resulting in antitumor activity.

Discussion

In this study, we showed that HGF cooperated with VEGF to enhance progression of *in vitro* angiogenesis, and that this process was resistant to treatment with lenvatinib, a potent inhibitor of VEGFRs. We showed that combined treatment with lenvatinib and golvatinib, an inhibitor of the HGF receptor, Met, rescued HUVECs and xenografted tumors from HGF pathway-induced resistance to lenvatinib.

Hepatocyte growth factor is produced from multiple non-tumor cell types under tumor microenvironments (e.g. cancer-associated fibroblasts), and participates in cancer progression; but some tumor cell types also secrete HGF and promote angiogenesis, migration, invasion, and metastasis of tumor cells in an autocrine and paracrine manner.⁽²⁸⁾ The melanoma cell line SEKI secretes high levels of both VEGF and HGF, and we

found that conditioned medium from cultured SEKI cells dramatically enhanced the proliferation of HUVECs *in vitro*. This enhancement was not inhibited by the VEGFR inhibitor lenvatinib or HGF pathway inhibitors, such as golvatinib or anti-HGF antibody. However, combined treatment with lenvatinib and HGF pathway inhibitors showed significant antiproliferative activity against SEKI conditioned medium-driven proliferation of HUVECs, suggesting that VEGF and HGF secreted from SEKI cells cooperated to promote the proliferation of HUVECs.

We observed that single treatment with lenvatinib or golvatinib showed limited or no antitumor activity against *in vivo* tumor growth in xenograft models derived from four HGF-producing human cancer cell lines (SEKI, IM95m, KP-4, and A2780) with distinct tissue origins. In contrast, combined treatment with lenvatinib and golvatinib significantly improved antitumor activity in all four models. As neither lenvatinib nor golvatinib showed a direct antiproliferative effect on the four human cancer cell lines *in vitro*, the results suggested that the VEGF and HGF produced by the cancer cells cooperated to promote tumor angiogenesis, and that this angiogenesis was

resistant to single treatment with VEGFR inhibitor but not combined treatment with lenvatinib and golvatinib. Supporting this notion, immunohistochemical staining of tumor endothelial cells with anti-CD31 antibody showed decreased numbers of tumor microvessels after the combined treatment, but not after single treatment with lenvatinib or golvatinib. Hepatocyte growth factor has also been implicated in resistance to VEGF/VEGFR signaling pathway inhibitors (e.g. bevacizumab and sorafenib) in clinical settings,^(18,19) and combination therapy with VEGFR and HGF pathway inhibitors may be effective in such HGF-overexpressing patients. However, combination therapy with lenvatinib and golvatinib did not completely suppress tumor growth in the xenograft models, and residual tumor blood vessels were evident after therapy. Therefore, alternative pathways other than VEGF/VEGFR2 signaling and HGF/Met signaling, such as delta-like 4/Notch signaling, may function in a compensatory manner,⁽⁹⁾ or some other mechanism may be involved.

Inhibition of the VEGF/VEGFR signaling pathway induces hypoxia of the tumor microenvironment, which in turn induces Met expression in tumor cells.^(29,30) The resultant Met induc-

tion has been reported to induce the epithelial–mesenchymal transition of tumor cells and to enhance the invasive and metastatic potential of tumor cells.^(30,31) Because lenvatinib is a potent VEGFR inhibitor, its potential to cause such phenomena cannot be ruled out. However, because the VEGF pathway inhibitor-induced enhancement of invasion and metastasis depends on an HGF-induced hypoxic microenvironment, the combination of lenvatinib with golvatinib may be expected to block this process, and further investigation is warranted.

Here we showed that VEGF and HGF cooperate to induce tumor angiogenesis in HGF-overexpressing cancer cells, HGF contributes to resistance to lenvatinib inhibition of angiogenesis, and combined use of golvatinib with lenvatinib overcomes this resistance. A clinical trial of combination therapy with lenvatinib and golvatinib (NCT01433991) is currently underway to assess both the combinational safety of lenvatinib and golvatinib and their efficacy in cancer patients.

Disclosure Statement

All authors are employees of Eisai Co, Ltd., Japan.

References

- Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 2011; **473**: 298–307.
- Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007; **6**: 273–86.
- Kim KJ, Li B, Winer J et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 1993; **362**: 841–4.
- Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov* 2011; **10**: 417–27.
- Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 2005; **8**: 299–309.
- Shojaei F, Wu X, Zhong C et al. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 2007; **450**: 825–31.
- Fischer C, Jonckx B, Mazzone M et al. Anti-PIGF inhibits growth of VEGF (R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 2007; **131**: 463–75.
- Crawford Y, Kasman I, Yu L et al. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* 2009; **15**: 21–34.
- Li JL, Sainson RC, Oon CE et al. DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy in vivo. *Cancer Res* 2011; **71**: 6073–83.
- Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 2008; **8**: 592–603.
- Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 2012; **12**: 89–103.
- Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Lett* 2005; **225**: 1–26.
- Peruzzi B, Bottaro DP. Targeting the c-Met signaling pathway in cancer. *Clin Cancer Res* 2006; **12**: 3657–60.
- Yano S, Wang W, Li Q et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008; **68**: 9479–87.
- Yamada T, Takeuchi S, Nakade J et al. Paracrine receptor activation by micro-environment triggers bypass survival signals and ALK inhibitor resistance in EML4-ALK lung cancer cells. *Clin Cancer Res* 2012; **18**: 3592–602.
- Wilson TR, Fridlyand J, Yan Y et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature* 2012; **487**: 505–9.
- Straussman R, Morikawa T, Shee K et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* 2012; **487**: 500–4.
- Miyahara K, Nouse K, Tomoda T et al. Predicting the treatment effect of sorafenib using serum angiogenesis markers in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 2011; **26**: 1604–11.
- Kopetz S, Hoff PM, Morris JS et al. Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J Clin Oncol* 2010; **28**: 453–9.
- 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.
- Nakagawa T, Tohyama O, Yamaguchi A et al. E7050: a dual c-Met and VEGFR-2 tyrosine kinase inhibitor promotes tumor regression and prolongs survival in mouse xenograft models. *Cancer Sci* 2010; **101**: 210–5.
- Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; **52**: 2745–56.
- Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* 2006; **1**: 1112–6.
- Dalal S, Berry AM, Cullinane CJ et al. Vascular endothelial growth factor: a therapeutic target for tumors of the Ewing's sarcoma family. *Clin Cancer Res* 2005; **11**: 2364–78.
- Tomida M, Saito T. The human hepatocyte growth factor (HGF) gene is transcriptionally activated by leukemia inhibitory factor through the Stat binding element. *Oncogene* 2004; **23**: 679–86.
- Jin H, Yang R, Zheng Z et al. MetMab, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. *Cancer Res* 2008; **68**: 4360–8.
- Iwai M, Matsuda M, Iwai Y. Cloning of a cancer cell-producing hepatocyte growth factor, vascular endothelial growth factor, and interleukin-8 from gastric cancer cells. *In Vitro Cell Dev Biol Anim* 2003; **39**: 288–90.
- Matsumoto K, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor–stromal interactions. *Int J Cancer* 2006; **119**: 477–83.
- Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met proto-oncogene. *Cancer Cell* 2003; **3**: 347–61.
- Sennino B, Ishiguro-Oonuma T, Wei Y et al. Suppression of tumor invasion and metastasis by concurrent inhibition of c-Met and VEGF signaling in pancreatic neuroendocrine tumors. *Cancer Discov* 2012; **2**: 270–87.
- Cooke VG, LeBleu VS, Keskin D et al. Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell* 2012; **21**: 66–81.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) production in cancer cells treated with lenvatinib or golvatinib.

Fig. S2. Expression of Met and signal transduction events in cancer cells treated with the Met inhibitor golvatinib.

Fig. S3. Relative body weight of mice during treatment with lenvatinib or golvatinib or both.

Fig. S4. Effect of combined lenvatinib and golvatinib treatment on hepatocyte growth factor-expressing human cancer xenograft models in nude mice.