

Anti-Vascular Endothelial Growth Factor Receptor-1 Antagonist Antibody as a Therapeutic Agent for Cancer

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Abstract Purpose: Vascular endothelial growth factor receptor-1 (VEGFR-1) plays important roles in promotion of tumor growth by mediating cellular functions in tumor vascular endothelium and cancer cells. Blockade of VEGFR-1 activation has been shown to inhibit pathologic angiogenesis and tumor growth, implicating VEGFR-1 as a potential therapeutic target for the treatment of cancer. We have thus developed a VEGFR-1 antagonist human monoclonal antibody designated as IMC-18F1 and evaluated its antitumor activity in preclinical experimental models to show the therapeutic potential of the antibody for cancer treatment in clinic.

Experimental Design: Human IgG transgenic mice were used for generation of anti-VEGFR-1 antibodies. Anti-VEGFR-1-specific blocking antibodies were identified using solid-phase binding and blocking assays. Inhibitory antitumor cell activity of IMC-18F1 was assessed in cell-based kinase and growth assays. Pharmacokinetic/pharmacodynamic studies were done to determine the association of antibody blood level with antitumor efficacy of the antibody *in vivo*. Antitumor efficacy of the anti-VEGFR-1 antibodies as monotherapy and in combination with cytotoxic agents was evaluated in human breast cancer xenograft models.

Results: A fully human neutralizing antibody, IMC-18F1, was shown to be a high-affinity ($K_D = 54$ pmol) inhibitor of VEGFR-1 ligand binding (VEGF-A, VEGF-B, and placental growth factor). IMC-18F1 inhibited ligand-induced intracellular activation of VEGFR-1 and mitogen-activated protein kinase signaling and prevented ligand-stimulated *in vitro* growth of breast cancer cells. *In vivo*, IMC-18F1 suppressed the growth of human breast tumor xenografts in association with reduced mitogen-activated protein kinase and Akt activation, reduced tumor cell proliferation, and increased tumor cell apoptosis. Pharmacokinetic/pharmacodynamic studies established a plasma elimination half-life of 5 days for IMC-18F1 and a steady-state trough plasma therapeutic threshold of 88 $\mu\text{g/mL}$. Importantly, inhibition of mouse and human VEGFR-1 with MF1 and IMC-18F1, respectively, enhanced the antitumor efficacy of cytotoxic agents commonly used to treat breast cancer.

Conclusions: Based on preclinical validation studies, IMC-18F1 anti-VEGFR-1 has potential to provide clinical benefit to cancer patients.

Target-directed therapies with monoclonal antibodies (mAb) and small-molecule inhibitors have improved therapeutic outcomes in cancer patients when combined with cytotoxic agents or radiation therapy. Anti-epithelial growth factor receptor antibodies that specifically prevent aberrant intracellular signaling activities for tumor cell survival and proliferation are efficacious as monotherapy and able to potentiate

antitumor efficacy of chemotherapy in patients with cancer (1). Vascular endothelial growth factor (VEGF)-A neutralizing antibody that blocks VEGF-A signaling for tumor angiogenesis has been used for treatment of patients with colon cancer, supporting VEGF/VEGF receptor (VEGFR) signaling pathways as promising targets for cancer intervention (2).

VEGF-A is a vital growth factor that mediates tumor-associated angiogenesis to support survival and growth of tumor cells and distant metastasis (3–5). VEGF-A regulates angiogenesis by activating two major transmembrane receptors, fms-like tyrosine kinase-1 (Flt-1, VEGFR-1) and kinase insert domain-containing receptor (KDR/Flk-1, VEGFR-2). Extensive studies in the past years have illustrated that VEGF-A induces proliferation, migration, and survival of endothelial cells via VEGFR-2 activation during angiogenesis process (4, 5). VEGFR-1 was initially found to play a role in the assembly of endothelial cells and modulation of blood vessel maturation during vasculogenesis and angiogenesis (6, 7). Studies in recent years have shown that VEGFR-1 is also present and functional

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in smooth muscle cells, monocytes, and hematopoietic stem/progenitor cells (8–10). In pathologic conditions, activation of VEGFR-1 has been found to promote tumor angiogenesis and metastasis through diverse mechanisms. VEGFR-1 activation was reported to stimulate angiogenesis via recruitment of endothelial and monocyte progenitor cells from bone marrow into tumor vasculature (10). Placental growth factor (PlGF), an exclusive ligand for VEGFR-1, was shown to act as a proangiogenic regulator and a survival factor for endothelial cells and macrophages supporting tumor angiogenesis (11). Deficiency of PlGF attenuated tumor angiogenesis *in vivo* (12, 13). VEGF-B, another VEGFR-1-specific ligand, was found to regulate angiogenesis through VEGFR-1-mediated activation of Akt and eNOS-related signaling pathways (14). VEGF-B expression was reported to be associated with lymph node metastasis in breast cancer (15). Hiratsuka et al. (16) showed that VEGFR-1 activation markedly promoted pulmonary metastasis through induction of matrix metalloproteinase-9 secretion in premetastatic lung endothelial cells and macrophages. We and others have shown that the blockade of VEGFR-1 activity toward angiogenesis by using an anti-mouse VEGFR-1 antibody MF1 inhibited tumor angiogenesis in tumor xenografts (13, 17). Most recently, Kaplan et al. (18) have shown the role of VEGFR-1 in the establishment of premetastatic niches promoting tumor metastasis.

Although VEGFR-1 is functionally expressed in various normal cell types that are involved in angiogenesis and metastasis, VEGFR-1 is also detected in cancer cells of a wide variety of tumor types, including leukemia, lymphoma, multiple myeloma, melanoma, and carcinomas of breast, colon, lung, pancreas, and prostate (17–28). VEGF-A stimulation induced activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/Akt signaling in VEGFR-1-positive breast cancer cells, resulting in increased invasiveness of the cancer cells (22). Studies have shown that stimulation with VEGF-A or VEGF-B activates MAPK, stress-activated protein kinase/c-Jun NH₂-terminal kinase, and nuclear factor- κ B in VEGFR-1-expressing colorectal cancer cells and induces *in vitro* growth of colonies of colon cancer cells and motility and invasiveness of colon and pancreatic carcinoma cells (23, 24). We have recently reported that VEGFR-1 plays a role in promoting the growth of breast cancer cells through activation of MAPK and Akt signaling in cancer cells, and treatment of mice with a mouse neutralizing anti-human VEGFR-1 antibody results in suppression of the growth of human breast tumor xenografts (25).

The findings in these studies illustrate that VEGFR-1 plays a significant role in regulating cellular functions in cancer cells as well as angiogenic cells to promote angiogenesis and tumor growth, and targeting VEGFR-1 may have therapeutic potential for the treatment of cancer. In the present study, we report the development of a fully human neutralizing mAb specific for human VEGFR-1 and its antitumor activity in preclinical models. This high-affinity antibody is designated as IMC-18F1 that specifically binds to human VEGFR-1 and blocks ligand binding to the receptor. We assessed the ability of the antibody to inhibit biological activities in VEGFR-1-expressing endothelial and human breast cancer cells and evaluated the antitumor activity of IMC-18F1 as monotherapy and in combination treatment with chemotherapies in the human breast carcinoma xenograft models. The results of these studies

further support the notion that VEGFR-1 is a valid therapeutic target for cancer intervention, and the neutralizing anti-VEGFR-1 antibody IMC-18F1 has a potential as a novel therapeutic means for treating patient populations with breast cancer and other tumor types.

Materials and Methods

Materials. All reagents and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Human VEGF-A (VEGF₁₆₅) and soluble recombinant human VEGFR-1 alkaline phosphatase and recombinant human VEGFR-2 alkaline phosphatase proteins were expressed in stably transfected cells and purified from cell culture supernatant following the procedures described previously (25). PlGF and soluble mouse recombinant VEGFR-1-Fc and human recombinant VEGFR-1-Fc proteins were purchased from R&D Systems (Minneapolis, MN). Cell cultureware and assay plates were purchased from BD Biosciences (Bedford, MA).

Cell lines. The human breast cancer cell lines DU4475, MDA-MB-231, and MDA-MB-435 and mouse myeloma cell lines P3-X63-Ag8.653 and NS0 were obtained from the American Type Culture Collection (Manassas, VA). The tumor cells were maintained in RPMI 1640 (Invitrogen/Life Technologies, Inc., Rockville, MD) containing 10% FCS (Hyclone, Logan, UT). Porcine aorta endothelial VEGFR-1 (PAE-VEGFR-1)-expressing cell line was provided by Dr. Lena Claesson-Welsh (Uppsala University, Uppsala, Sweden) and cultured in F-12 medium (Invitrogen/Life Technologies) containing 10% FCS. All cells were maintained at 37°C in a humidified, 5% CO₂ atmosphere.

Generation of human anti-VEGFR-1 antibodies. Human anti-human VEGFR-1 mAbs were generated by a standard hybridoma technology using KM human IgG transgenic mice (Medarex, San Jose, CA), which produce human immunoglobulin γ heavy and κ light chain. KM mice were immunized s.c. with VEGFR-1-Fc in complete Freund's adjuvant. Animals were i.p. boosted thrice with the VEGFR-1 protein in incomplete Freund's adjuvant. The mice were rested for a month before they received the final i.p. boost of VEGFR-1 protein 4 days before fusion. Splenocytes were harvested from the immunized mouse and fused with myeloma cells using polyethylene glycol. After fusion, the cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and hypoxanthine, aminopterin, and thymidine in 96-well plates. Hybridomas producing anti-human VEGFR-1 mAbs were screened for specific binding and blocking activity of culture supernatant in ELISA-based binding and blocking assays as described previously (18). The positive hybridomas were subcloned thrice by a limiting dilution culture for establishment of monoclonal hybridomas.

Cloning and sequencing of VH/VL regions of anti-VEGFR-1 antibodies. Polyadenylated mRNA was isolated from hybridoma cells derived from VEGFR-1 immunized KM mice using a FastTrack kit (Invitrogen, Carlsbad, CA). The generation of random-primed cDNA was followed by PCR using a Clontech (Mountain View, CA) kit. Primers 5'-ATGGAGTTGGGCTGAGCTG (forward) and 3'-TGCCAGGGGAAGACCGATGG (reverse) and 5'-ATGGAACCCAGCGCAGCTTCTC (forward) and 3'-CGGGAAGATGAAGACAGATG (reverse) were used for binding to variable regions of heavy and κ light chains, respectively. Sequences of human immunoglobulin-derived heavy and κ chain transcripts from hybridomas were obtained by direct sequencing of PCR products generated from polyadenylated RNA using the primers described above. PCR products were also cloned into pCR2.1 using a TA cloning kit (Invitrogen), and both strands were sequenced using Prism dye terminator sequencing kits and an ABI 3730 sequencer (Genewiz, North Brunswick, NJ). All sequences were analyzed by alignments to the Kataman antibody sequence program using the DNASTAR software.

Engineering and expression of human IgG1 anti-VEGFR-1 antibodies. The DNA sequences encoding the heavy and light chain genes of variable regions of the anti-VEGFR-1 antibodies were amplified by PCR for cloning into expression vectors. The heavy chain variable regions were fused in frame to the human immunoglobulin heavy chain γ 1 constant region in vector pEE6.1 (Lonza Biologics plc, Slough, Berkshire, United Kingdom). The entire human light chain cDNA was cloned directly into vector pEE12.1 (Lonza Biologics plc). Engineered immunoglobulin expression vectors were stably transfected in NS0 myeloma cells by electroporation and selected in glutamine synthetase selection medium. Stable clones were screened for antibody expression by anti-Fc and VEGFR-1-specific binding ELISA. Positive clones were cultured into serum-free medium culture for antibody production in spinner flasks or bioreactors. Full-length IgG1 antibody was purified by protein affinity chromatography (Poros A, PerSeptive Biosystems, Inc., Foster City, CA) and eluted into a neutral-buffered saline solution.

Measurement of affinity of human VEGFR-1 antibody. Affinities of purified anti-VEGFR-1 antibodies were determined by plasmon resonance technology using BIAcore 2000 instrument (Pharmacia, Piscataway, NJ) according to the procedures provided by the manufacturer. Kinetic analyses of the antibodies were done by immobilization of recombinant extracellular domain of VEGFR-1 onto the sensor surface at a low density. The association (K_{on}) and dissociation (K_{off}) rates were determined using the BIAevaluation 2.1 software provided by the manufacturer.

Solid-phase receptor binding and blocking assay. Human VEGFR-1-Fc, mouse VEGFR-1-Fc, or human VEGFR-2 alkaline phosphatase was coated at 100 ng/well on 96 microtiter plates and blocked with the blocking buffer of PBS-0.05% Tween 20 containing 5% dry milk (Bio-Rad Laboratories, Hercules, CA). The binding activity of anti-human VEGFR-1 antibodies and anti-mouse VEGFR-1 antibody MF1 to VEGFR-1 or VEGFR-2 was evaluated as described previously (17), except that bound antibody was detected with a goat anti-human κ -horseradish peroxidase antibody (BioSource International, Camarillo, CA) for IMC-18F1 and anti-human VEGFR-2 antibody 1C11 or a goat anti-rat IgG-horseradish peroxidase antibody (BioSource International) for MF1. For blocking assay, human VEGF-A, VEGF-B, PlGF, or the mixture of all three ligands was coated at 200 ng/well on 96-well plates, and wells were then blocked with the blocking buffer. A serial dilution of VEGFR-1 antibody was preincubated with recombinant human VEGFR-1 alkaline phosphatase for 1 hour in 96-well plates. The solution of the reaction was then transferred to the ligand-coated 96-well plates and incubated for 1 hour. After washing, *p*-nitrophenyl phosphate substrate for alkaline phosphatase was added to the wells for color development. The absorbance at 405 nm was read for quantification of VEGFR-1 binding activity with ligands. Data were analyzed by using GraphPad Prism software 3.03 (GraphPad Software, Inc., San Diego, CA).

Surface native VEGFR-1 blocking assay. The binding of 125 I-VEGF to native VEGFR-1 on cell surface was done using PAE-VEGFR-1-expressing cells. Briefly, the cells were grown on noncoated plastic cell culture plates in which it was found to decrease nonspecific binding without affecting the specific binding of 125 I-VEGF. Confluent cells were incubated in serum-free and growth supplement-free DMEM/F-12 medium for 24 hours. Cells were rinsed once with ice-cold DMEM/F-12 medium containing 25 mmol/L HEPES and 1 mg/mL bovine serum albumin (BSA). A serial dilution of anti-VEGFR-1 antibody or cold VEGF at the concentration of a 200-fold molar excess of the labeled VEGF was added to each well in the plate and incubated at 4°C for 1 hour. After wash, 125 I-VEGF was added at the concentration of 2 ng/mL and incubated at 4°C for 2 hours on a platform shaker. The cells were washed thrice with PBS containing 1 mg/mL BSA and 0.25 mmol/L CaCl₂ and incubated for 5 minutes in the presence of 1% Triton X-100, 1 mg/mL BSA, and 0.16% NaN₃ to remove bound VEGF. The soluble content of each well was counted in a gamma counter. The assays were done in triplicate in at least three independent experiments, and the data were analyzed using GraphPad Prism software 3.03.

Flow cytometry analysis. Aliquots of 5×10^5 PAE-VEGFR-1 transfectant cells and DU4475, MDA-MB-231, and MDA-MB-435 breast carcinoma cells were harvested from subconfluent cultures and incubated with anti-VEGFR-1 antibody IMC-18F1 in PBS with 1% BSA and 0.02% sodium azide (staining buffer) for 1 hour on ice. A matched IgG isotype (Jackson ImmunoResearch, West Grove, PA) was used as a negative control. Cells were washed twice with the staining buffer and then incubated with a FITC-labeled goat anti-human IgG antibody (BioSource International) in the buffer for 30 minutes on ice. Cells were washed as above and analyzed on an Epics XL flow cytometer (Beckman Coulter, Hialeah, FL). Dead cells and debris were eliminated from the analysis based on forward and sideways light scatter. The mean fluorescent intensity ratio (MFIR) was calculated to quantitate relative expression levels of VEGFR-1 in the cell lines. The MFIR is the MFI of cells stained with VEGFR-1-specific mAb divided by the MFI of cells stained with an isotype control mAb.

Immunoprecipitation and Western blot analysis. PAE-VEGFR-1 cells were plated into 100-mm culture dishes and grown to 70% to 80% confluence. Monolayers were washed twice with PBS and cultured overnight in serum-free medium. After replacing the culture medium, the cells were treated with IMC-18F1 or isotype control at 37°C for 1 hour and incubated with 50 ng/mL VEGF-A or 100 ng/mL PlGF for 10 to 15 minutes. After washed with ice-cold PBS, the cells were lysed with 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.5 mmol/L sodium orthovanadate, 50 mmol/L HEPES (pH 7.4), 1 mmol/L phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN) on ice for 20 minutes. The lysate was clarified by centrifugation at 4°C. Solubilized VEGFR-1 was immunoprecipitated from the lysate using anti-VEGFR-1 antibody (C-17, Santa Cruz Biotechnology, Santa Cruz, CA) and protein A agarose beads at 4°C. The immune complex was subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Phosphorylated VEGFR-1 was detected with an anti-phosphorylated kinase antibody (PY-20, Santa Cruz Biotechnology). Phosphorylated-specific Akt (Ser⁴⁷³), total Akt, phosphorylated-specific p44/42 (Thr²⁰²/Tyr²⁰⁴) MAPK, and total p44/42 MAPK were determined using respective primary antibodies (Cell Signaling Technology, Beverly, MA). The specific protein signals were visualized on X-ray film (Eastman Kodak, Rochester, NY) after incubation of blotted membranes with horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Cell growth assay. DU4475 carcinoma cells (2×10^4 per well) were seeded in 96-well plates and incubated in serum-free conditions for 18 hours and then cultured in 1% serum-containing medium with IMC-18F1 or isotype control IgG at 22, 66, or 200 nmol/L in the presence of 50 ng/mL VEGF or 200 ng/mL PlGF for 48 hours. For hypoxia induction, cells were treated with 100 nmol/L desferrioxamine for 5 hours before antibody treatment. Viable cells were counted in triplicate using a Coulter cytometer (Coulter Electronics Ltd., Luton, Beds, United Kingdom). Each experiment was done in triplicate. The following formula was used for calculation of percentages of the control: %Control = [(Tx - To) / (GC - To)] \times 100, where Tx is antibody treated, To is untreated background, and GC is growth control (i.e., VEGF-A-stimulated or PlGF-stimulated cell growth).

Antibody internalization assay. Antibody IMC-18F1 was radioiodinated with 125 I (Amersham Pharmacia Biotech) per manufacturer's instructions. PAE-VEGFR-1 cells were plated into six-well plates and cultured overnight to 70% confluence. A serial dilution of 125 I-labeled or unlabeled IMC-18F1 was added to each well and incubated at 37°C or kept on ice at 4°C for 30, 60, and 120 minutes, and each time point was done in triplicate. The control was held at 4°C for 90 or 120 minutes. Wells were washed thrice with PBS/0.2% BSA and then stripped for 5 minutes with 100 mmol/L glycine-HCl and 2 mol/L urea (pH 2.5). Cells were washed thrice with PBS/0.2% BSA and solubilized with 1 N NaOH/1% Triton X-100. Stripped and solubilized fractions were read on a gamma counter. To ensure the specificity of the

IMC-18F1-induced internalization, the quantity of internalized ^{125}I -IMC-18F1 was calculated using the formula of counts per minute (cpm) counts of bound ^{125}I -IMC-18F1 subtracted by the cpm of ^{125}I -IMC-18F1 incubated with cold IMC-18F1-pretreated cells.

Pharmacokinetic/pharmacodynamic studies with MF1 and IMC-18F1. Pharmacokinetic studies were done in naive C57BL/6J female mice, and pharmacokinetic/pharmacodynamic studies were done in female athymic *nu/nu* mice bearing DU4475 or MDA-MB-231 breast cancer xenografts established as described below. In the pharmacokinetic/pharmacodynamic studies, once tumors reached approximately 200 to 300 mm³, mice were randomized to receive IMC-18F1 at different doses. Control mice received human IgG (Biodesign International, Saco, ME) and/or USP saline. For the first treatment, loading doses (1.8 times the maintenance dose) were calculated using the plasma elimination half-life measured in the single-dose pharmacokinetic study, the maintenance dose, and the dosing interval to bring the predicted plasma antibody level immediately to steady state. Mice were bled up to 14 days after dosing for the pharmacokinetic study and up to 72 hours after the final dose for the pharmacokinetic/pharmacodynamic study ($n = 2-4$ per time point and treatment group). Plasma IMC-18F1 concentrations were determined by ELISA using goat anti-human IgG (Sigma) as the capture antibody and horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch) as the detection antibody. IMC-18F1 was used to prepare the standard curve for the ELISAs.

From the single-dose pharmacokinetic study, measured IMC-18F1 and MF1 plasma concentrations were used to calculate steady pharmacokinetic variables using a one-compartment, first-order input pharmacokinetic model with WinNonlin 4.0.1 software (Pharsight Corp., Mountain View, CA). The WinNonlin pharmacokinetic variables determined from this analysis were $V_F = 104.714$ mL/kg, $K_{01} = 0.442$ /hour, and $K_{10} = 0.0091$ /hour. These pharmacokinetic variables were then used to predict plasma IMC-18F1 concentration throughout the pharmacokinetic/pharmacodynamic study assuming pharmacokinetic linearity, and predictions were compared with the steady-state measured values.

Treatment of human breast carcinoma xenografts. Athymic nude mice (Charles River Laboratories, Wilmington, MA) were injected s.c. in the left flank area with 2×10^6 of DU4475 cells and 5×10^6 of MDA-MB-231 or MDA-MB-435 cells mixed in Matrigel (Collaborative Research Biochemicals, Bedford, MA). Tumors were allowed to grow ~ 200 mm³ in size, and mice were then randomized into groups of 12 animals per group. For MDA-MB-435 model, the tumor cells were implanted s.c. into mammary fat pad area in mice. Animals received i.p. administration of IMC-18F1 at a dose of 20 or 40 mg/kg twice or thrice weekly. In the antibody combination treatment, mice bearing established tumors were treated with 20 mg/kg IMC-18F1 twice weekly and 40 mg/kg MF1 thrice weekly. IMC-18F1 was injected 1 day after treatment with MF1. For combination treatment with antibody and chemotherapy, tumors were allowed to grow ~ 300 mm³, mice were then randomized into groups of 12 animals per group, and a cocktail of 20 mg/kg IMC-18F1 and 40 mg/kg MF1 was administered i.p. 1 or 2 days before chemotherapy with 125 mg/kg 5-fluorouracil and 62 mg/kg leucovorin (5-FU/LV) q7d, 3 mg/kg doxorubicin twice weekly, or 100 mg/kg cyclophosphamide q7d. Mice in control groups received vehicle or normal IgG as a negative control. Tumors were measured twice weekly with calipers. Tumor volumes were calculated using the formula $\pi / 6 (w_1 \times w_2 \times w_2)$, where "w1" represents the largest tumor diameter and "w2" represents the smallest tumor diameter. All animal studies were conducted in accordance with the guidelines of the NIH "Guide for Care and Use of Animals" and an approved protocol reviewed by the company's Institutional Animal Care and Use Committee.

Immunohistochemical analysis of human breast xenografts. Paraffin-embedded MDA-MB-231 xenografts were evaluated immunohistochemically for markers of cell proliferation, survival, and apoptosis. Antibodies to Ki-67 (Lab Vision Corp., Fremont, CA), phosphorylated-

specific p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴; Cell Signaling Technology), and phosphorylated-specific Akt (Ser⁴⁷³; Cell Signaling Technology) were used as primary antibodies. The EnVision+ System for rabbit antibodies (DakoCytomation, Carpinteria, CA) was used with 3,3'-diaminobenzidine as the chromagen per kit instructions. After brief counterstaining in Mayer's hematoxylin, all sections were dehydrated, cleared, and coverslipped using a permanent mounting medium. Tumor apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using ApopTag Peroxidase *In situ* Apoptosis Detection kit (Chemicon, Temecula, CA) per kit instructions. Stained sections were coverslipped with GelMount (Biomedica, Foster City, CA). Positive immunostaining and TUNEL-positive immunofluorescence were analyzed and imaged using an Axioskop light microscope with an AxioCam digital camera (Carl Zeiss, Göttingen, Germany).

Statistical analysis. Tumor volume data were analyzed using repeated measures ANOVA to determine the significant differences in tumor sizes among treatments, time points, and treatment-time interactions. Comparisons of *in vitro* tumor cell growth between treatment and control were conducted using the two-tailed Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Results

Specificity and binding and blocking activity of the neutralizing human anti-VEGFR-1 antibody IMC-18F1. Four hybridoma clonal cell lines producing human anti-VEGFR-1 neutralizing mAbs were established. Variable region cDNA of the antibodies from these hybridomas were then cloned and engineered for expression in mammalian cells as full-length human IgG1 antibodies. Table 1 summarizes the binding and blocking characteristics of the anti-human VEGFR-1 neutralizing antibodies. Kinetic analysis on the BIAcore instrument indicated that IMC-18F1 possessed the strongest affinity binding ($K_D = 54$ pmol) to VEGFR-1 of all clones tested. A solid-phase binding assay showed that IMC-18F1 had exhibited strong binding activity ($ED_{50} = 0.1$ nmol/L) to recombinant human VEGFR-1 protein (Fig. 1A; Table 1) and did not cross-react with mouse VEGFR-1 (Fig. 1B) or KDR (data not shown), indicating a strict binding specificity of IMC-18F1 with human VEGFR-1. Fluorescence-activated cell sorting analysis showed that IMC-18F1 recognized native VEGFR-1 expressed on PAE-VEGFR-1 transfectant (MFIR = 6.8; Fig. 1C) and DU4475 breast carcinoma cell lines (MFIR = 3.4; Fig. 1D). A similar level of binding activity of IMC-18F1 with MDA-MB-231 and MDA-MB-435 carcinoma cell lines was also observed (data not shown). Solid-phase blocking assay showed that IMC-18F1 effectively blocked VEGFR-1 binding to VEGF-A, VEGF-B, and PlGF with IC_{50} of 1.04, 0.43, and 0.38 nmol/L, respectively (Fig. 1E-G; Table 1). The antibody also exhibited comparable blocking activity with the receptor binding to the mixture of VEGF-A, VEGF-B, and PlGF in the blocking assay where all three ligands were precoated together in plate (data not shown). In addition, IMC-18F1 significantly inhibited the ^{125}I -VEGF binding to the native VEGFR-1 on the porcine aorta endothelial cells with IC_{50} of 0.09 nmol/L (Fig. 1H) in the cell-based assay.

IMC-18F1 inhibits PlGF-induced autophosphorylation of VEGFR-1 and activation of MAPK. To assess inhibitory activity of IMC-18F1 in blocking autophosphorylation of VEGFR-1 and subsequent activation of downstream kinase signaling pathways, we did *in vitro* studies with IMC-18F1 in ligand-dependent assay on PAE-VEGFR-1 transfectant cells. As

Table 1. The binding and blocking characteristics of anti-human VEGFR-1 antibodies

Clone	Kinetics of anti-VEGFR-1 antibodies*			Binding activity [†] (ED ₅₀ , nmol/L)	Blocking activity [†] (IC ₅₀ , nmol/L)
	K _{on} 10 ⁵ M ⁻¹ s ⁻¹	K _{off} 10 ⁻⁵ s ⁻¹	K _D 10 ⁻¹¹ M		
6F9	11.1 ± 1.26	7.38 ± 2.01	6.9 ± 0.36	0.145 ± 0.03	VEGF-A: 1.05 ± 0.03 VEGF-B: ND PlGF: 0.81 ± 0.05
13G12	9.58 ± 1.63	11.05 ± 3.06	12.4 ± 0.53	0.303 ± 0.02	VEGF-A: 0.99 ± 0.02 VEGF-B: ND PlGF: 0.73 ± 0.04
15F11	10.16 ± 2.84	7.16 ± 2.21	7.0 ± 0.23	0.304 ± 0.01	VEGF-A: 0.82 ± 0.03 VEGF-B: ND PlGF: 0.48 ± 0.01
IMC-18F1	8.1 ± 2.4	4.29 ± 1.05	5.4 ± 0.3	0.112 ± 0.04	VEGF-A: 1.04 ± 0.01 VEGF-B: 0.43 ± 0.01 PlGF: 0.38 ± 0.02

Abbreviation: ND, not determined.

*The values of kinetics of the antibodies were calculated from the data determined by BIAcore analysis.

[†]The ED₅₀ and IC₅₀ values were calculated from the data analyzed using GraphPad Prism software. Binding activity was determined by measuring ability of the antibodies from binding to recombinant human VEGFR-1 in ELISA. Blocking activity was determined by measuring ability of the antibodies preventing recombinant human VEGFR-1 from binding to VEGF-A, VEGF-B, or PlGF in ELISA.

shown in Fig. 2A, stimulation with 100 ng/mL VEGFR-1-specific ligand PlGF resulted in a significant increase of phosphorylated VEGFR-1 in PAE-VEGFR-1 cells. Treatment with 100 nmol/L IMC-18F1 reduced VEGFR-1 activation induced by PlGF to basal levels. Significant phosphorylation of MAPK was also induced by PlGF in PAE-VEGFR-1 transfectant cells (Fig. 2B). Treatment with IMC-18F1 (>10 nmol/L) resulted in the decrease of PlGF-induced phosphorylation of MAPK to basal levels. Isotype-matched normal IgG had no effect on PlGF-stimulated activation of VEGFR-1 and MAPK.

IMC-18F1 blocks in vitro growth of breast cancer cells. Ability of IMC-18F1 to inhibit ligand-dependent proliferation of

VEGFR-1-expressing breast carcinoma cells was assessed in a cell-based growth assay. It has been shown that hypoxia up-regulates a variety of genes implicated in the survival and invasive phenotype switch in tumor cells and that VEGFR-1 is up-regulated in endothelial cells in response to hypoxia (29). We evaluated whether treatment with IMC-18F1 inhibited VEGFR-1 ligand-induced proliferation of DU4475 breast carcinoma cells in hypoxic and normoxic conditions. The cell-based assay showed that the treatment of IMC-18F1 markedly blocked VEGF-A-stimulated (Fig. 3A) and PlGF-stimulated (Fig. 3B) proliferation of hypoxic tumor cells in a dose response. A similar result was observed in treatment of the

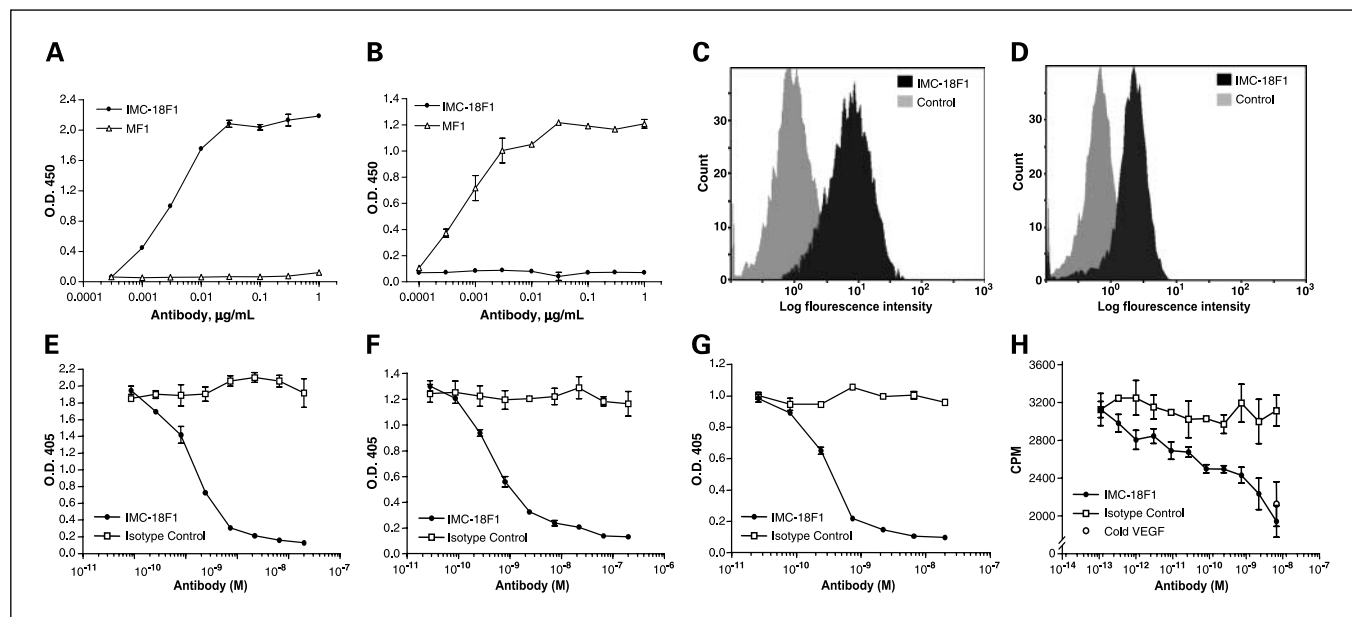


Fig. 1. Specificity and binding and blocking activity of IMC-18F1. IMC-18F1 binds to immobilized recombinant human VEGFR-1 (A) but not mouse VEGFR-1 (B) in ELISA binding assay. Flow cytometry shows the binding activity of IMC-18F1 with native VEGFR-1 on PAE-VEGFR-1 (MFIR = 6.8; C) and DU4475 (MFIR = 3.4; D) cells. As measured in ELISA blocking assay, IMC-18F1 effectively inhibited VEGF-A (E), VEGF-B (F), and PlGF (G) binding to VEGFR-1 with IC₅₀ of 1.04, 0.43, and 0.38 nmol/L, respectively. H, binding activity of ¹²⁵I-VEGF-A with VEGFR-1 on PAE-VEGFR-1-expressing cells was remarkably blocked by IMC-18F1 with IC₅₀ of 0.09 nmol/L. Isotype-matched antibody did not interfere with the ligand binding to VEGFR-1 in both assays.

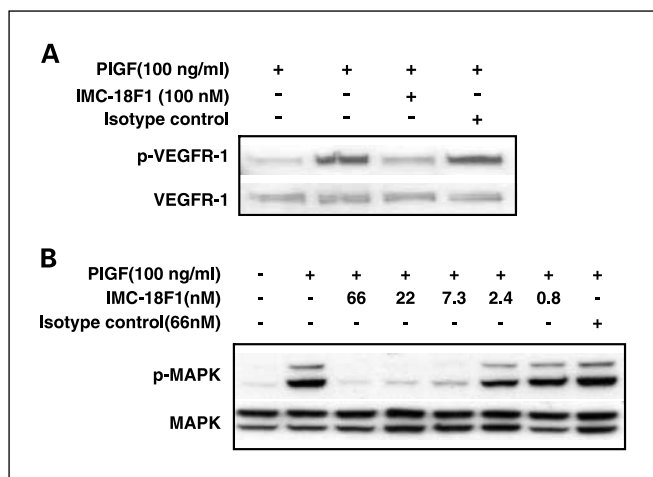


Fig. 2. Inhibition of PIGF-induced VEGFR-1 phosphorylation and MAPK activation. *A*, PIGF stimulation induced an increase in VEGFR-1 phosphorylation. IMC-18F1 effectively inhibited the PIGF-induced phosphorylation of VEGFR-1. Western blots were probed for phosphorylated VEGFR-1 (*p-VEGFR-1*; top) and reprobed with a VEGFR-1 antibody for the total receptor (*bottom*). *B*, PIGF stimulation activated p44/p42 MAPK. IMC-18F1 blocked the PIGF-stimulated activation of MAPK in a dose response. Western blots were probed for phosphorylated p44/p42 MAPK (*p-MAPK*; top) and reprobed with an antibody for total MAPK (*bottom*). Isotype control antibody had no effects.

tumor cells with IMC-18F1 in low-percentage serum culture under normoxic condition (data not shown).

IMC-18F1 mediates surface VEGFR-1 internalization. We did an assay to determine whether IMC-18F1 could mediate internalization of the bound receptor. As shown in Fig. 4, radiolabeled IMC-18F1 bound to the surface of PAE-VEGFR-1 cells and was internalized in a physiologic temperature condition. The VEGFR-1 antibody induced the down-modulation of the receptor occurred in a dose-dependent manner. A time course assay indicated that the antibody-induced internalization of the receptor reached maximum after 60 minutes of incubation (data not show). This shift was not observed when cells were incubated at 4°C with IMC-18F1 at a high dose, suggesting that the receptor internalization process was mediated through the engagement of VEGFR-1 binding-specific antibody with the receptor.

Pharmacokinetics and pharmacodynamics of IMC-18F1 and MF1. To determine the plasma IMC-18F1 concentrations necessary to elicit the direct effects on tumor cell growth in the xenograft models, we did pharmacokinetic and pharmacokinetic/pharmacodynamic studies. In a single i.p. dose pharmacokinetic study done in naive female C57BL/6J mice, IMC-18F1 had an elimination half-life of 4.8 days and a maximal plasma concentration of 203 µg/mL reached at 6 hours after dosing. When dosed twice weekly, with a loading dose of 1.8 times the maintenance dose in the MDA-MB-231 breast cancer xenograft model, IMC-18F1 at maintenance doses of 20 and 50 mg/kg had comparable antitumor activity (Fig. 5A). Below 20 mg/kg, efficacy significantly decreased. Similar to the results in the MDA-MB-231 model, IMC-18F1 dosed at 20 and 60 mg/kg maintenance dose twice weekly had comparable activity in the DU4475 xenograft model, whereas 6 mg/kg was without significant efficacy (data not shown). Steady-state pharmacokinetic measurements made after the eighth treatment in the MDA-MB-231 model (Fig. 5A) provided a therapeutic target for trough plasma

antibody concentration of 88 µg/mL in the 20 mg/kg treatment group ($n = 4$ trough measurements per treatment group). This trough value will be used to guide dosing in phase I clinical studies. Steady-state pharmacokinetic measurements were accurately predicted by using the single-dose pharmacokinetic measurements in a one-compartment, first-order input pharmacokinetic model assuming linearity (Fig. 5B). Therefore, the single-dose pharmacokinetic measurements can also be used to predict the plasma IMC-18F1 pharmacokinetic variables associated with efficacy in this model to compare with clinical measurements.

The single-dose pharmacokinetic study with MF1 done in C57BL/6J mice indicated that MF1 had an elimination half-life of 3.0 days and reached a maximal plasma concentration of 130 µg/mL at 6 hours after dosing. The result suggested that

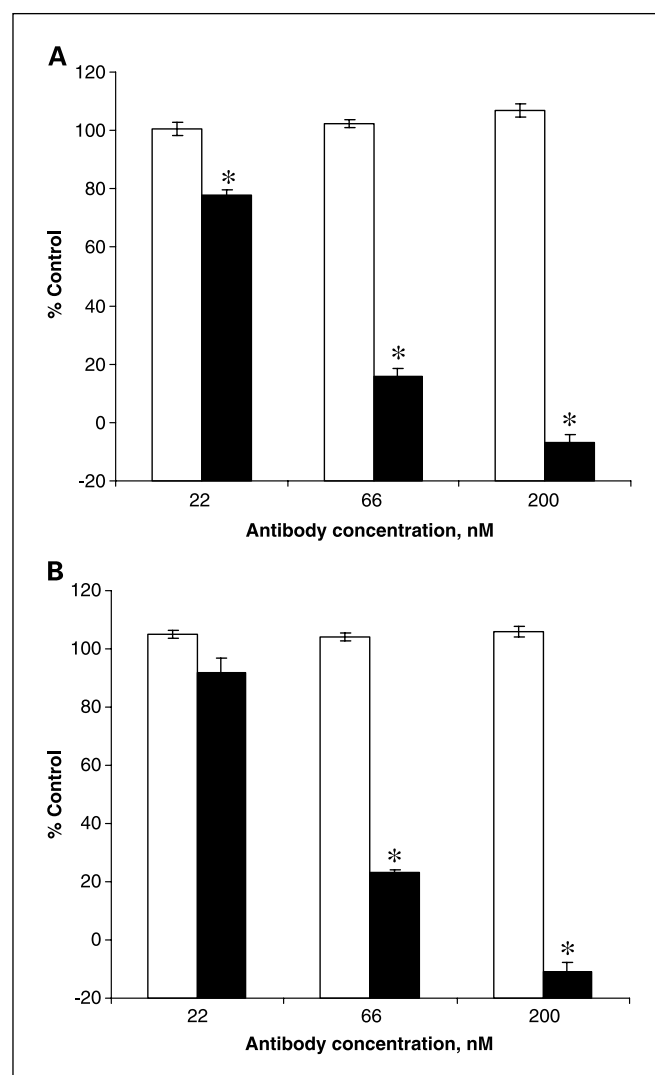


Fig. 3. Inhibition of tumor cell proliferation. Serum-starved DU4475 cells were treated with desferrioxamine followed by treatment with various concentration of IMC-18F1 (*black columns*) or isotype control IgG (*white columns*) in the presence of VEGF-A or PIGF. IMC-18F1 significantly inhibited proliferation of the tumor cells stimulated by VEGF-A (*A*) or PIGF (*B*) in a dose-dependent manner. Data are percentage of inhibition calculated by percentage control. Columns, mean; bars, SE. This was a representative experiment of three with similar results. *, $P < 0.05$, statistical significance as comparison between IMC-18F1 and isotype control antibody-treated groups.

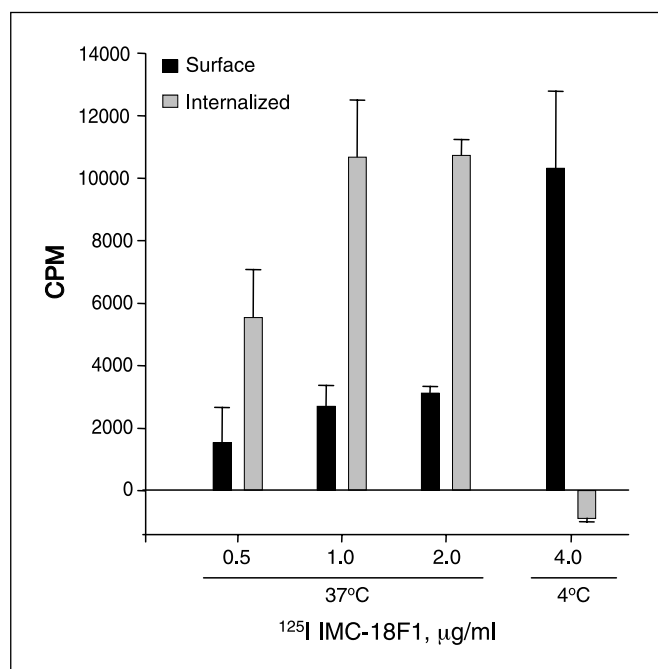


Fig. 4. Induction of receptor internalization. ^{125}I -radiolabeled or unlabeled IMC-18F1 at different dose was incubated with PAE-VEGFR-1 cells at 37°C or 4°C. At the times specified, cells were washed with PBS and membrane-bound antibody was stripped and collected for count. Cells were lysed and counted for representing internalized antibody. The quantity of internalized receptors was presented with cpm counts that was calculated using the formula of cpm of bound ^{125}I -IMC-18F1 subtracted by cpm of ^{125}I -IMC-18F1 incubated with cold IMC-18F1-pretreated cells. Columns, mean; bars, SE.

MF1 should be dosed twice or thrice weekly to maintain relatively stable blood level in mouse tumor models. In all studies, reported MF1 was dosed on a Monday-Wednesday-Friday schedule.

Treatment with IMC-18F1 suppresses in vivo growth of human breast carcinoma xenografts. The antitumor activity of IMC-18F1 was evaluated in human breast carcinoma xenograft models of DU4475, MDA-MB-231, and MDA-MB-435. Monotherapy studies with IMC-18F1 have indicated that dosing of IMC-18F1 twice weekly at 20 mg/kg is as effective as the dose at 40 mg/kg in MDA-MB-231 and MDA-MB-435 models (data not shown). For the aggressive DU4475 model, 40 mg/kg was chosen to assure effective inhibition of the tumor growth in the xenograft studies. The data from these studies and pharmacokinetic and pharmacodynamic analysis of IMC-18F1 and MF1 were used as guidance for dosing animals in xenograft studies. As shown in Fig. 6, systemic administration of IMC-18F1 twice weekly significantly suppressed the growth of tumor xenografts of MDA-MB-435 [% treated versus control (%T/C) = 47; $P < 0.01$; Fig. 6A], MDA-MB-231 (%T/C = 36; $P < 0.03$; Fig. 6B), and DU4475 (%T/C = 52; $P < 0.001$; Fig. 6C). To illustrate that the antitumor activity of IMC-18F1 was the results of inhibitory effects on intracellular kinase signaling pathways required for tumor cell proliferation and survival, we did histologic analysis on IMC-18F1-treated xenografts of MDA-MB-231 tumors. As shown in Fig. 7, the proliferative activity as marked with Ki-67 molecule and phosphorylation of MAPK and Akt were significantly reduced in tumor cells in IMC-18F1-treated tumor xenografts. In addition, increased tumor

cell apoptosis, as measured by TUNEL staining, was observed in the antibody-treated xenografts.

In vivo blockade of human and murine VEGFR-1 leads to enhanced antitumor activity against human breast carcinoma xenografts. IMC-18F1 is expected to have a dual antitumor and antiangiogenic activity through inhibition of VEGFR-1 activation in tumor cells as well as endothelial cells when the antibody is used to treat cancer patients. To show the dual antitumor mechanisms of anti-VEGFR-1 antibody in tumor xenograft models mimicking the clinical scenario, we did *in vivo* studies where mice bearing established tumors were concomitantly treated with IMC-18F1 and MF1, an anti-mouse VEGFR-1 antibody that suppresses tumor growth through inhibition of tumor angiogenesis (13). As shown in Fig. 6C, the cotreatment with IMC-18F1 and MF1 (40 mg/kg each, twice and thrice weekly, respectively) resulted in significantly increased antitumor effect on DU4475 xenografts (%T/C = 29; $P < 0.01$) compared with monotherapy with IMC-18F1 (%T/C = 52; $P < 0.001$ versus control) or MF1 (%T/C = 49; $P < 0.001$ versus control).

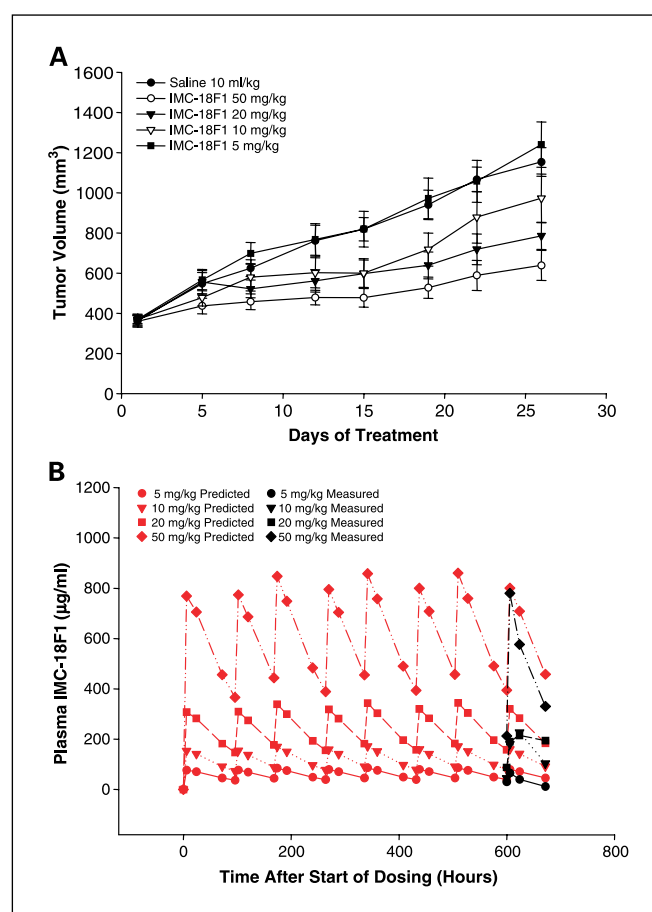


Fig. 5. Inhibition of the MDA-MB-231 tumor xenografts in IMC-18F1 pharmacokinetic/pharmacodynamic study. **A**, loading doses of 9, 18, 36, and 90 mg/kg were administered to mice bearing established tumors on the 1st day of treatment followed by maintenance doses of 5 (■), 10 (Δ), 20 (▲), and 50 mg/kg (○), respectively, on a twice weekly schedule in the MDA-MB-231 breast cancer xenograft model. Saline (●) was used for the treatment of control group on the same dosing schedule. Points, mean tumor volume; bars, SE. **B**, predicted (red symbols) and measured (black symbols) plasma IMC-18F1 concentration. Predicted values were calculated using a one-compartment, first-order input pharmacokinetic model and WinNonlin 4.0.1 software to model the single-dose pharmacokinetic data for IMC-18F1. Points, mean ($n = 2-4$) of measured values.

VEGFR-1 antibody potentiates antitumor activity of chemotherapy. To determine whether combining VEGFR-1 inhibition with cytotoxic agents used in the treatment of breast cancer leads to an enhanced antitumor effect, we tested antitumor

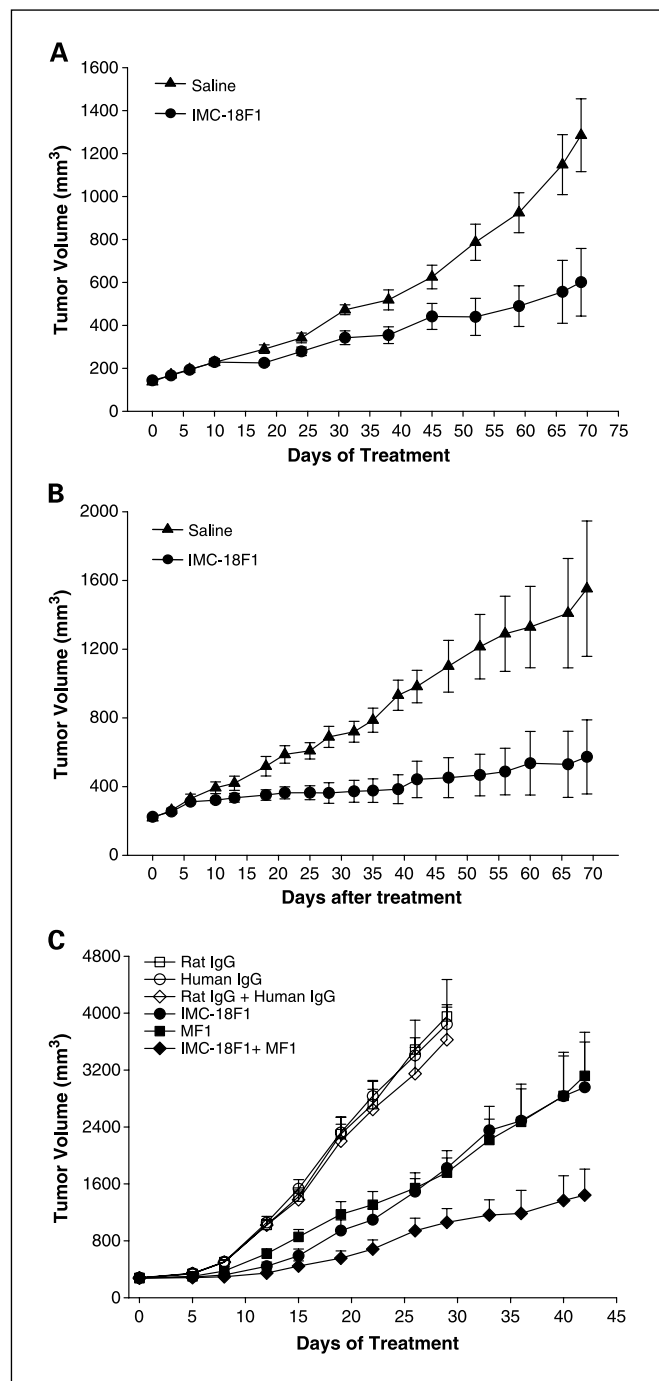


Fig. 6. Inhibition of human breast tumor xenografts. Monotherapy twice weekly with IMC-18F1 (●) at dose of 20 mg/kg for MDA-MB-435 (A) and MDA-MD-231 (B) and 40 mg/kg for DU4475 (C) significantly inhibited the growth of tumor xenografts compared with respective controls vehicle (PBS; A and B, ▲) or 20 mg/kg human normal IgG (C, ○). C, combination treatment with IMC-18F1 and MF1 (40 mg/kg each, twice and thrice weekly, respectively; ◆) resulted in a significantly increased antitumor activity ($P < 0.01$) compared with monotherapy with IMC-18F1 (●) or MF1 (■) in the DU4475 xenograft model. Mice treated with 20 mg/kg human IgG (□), 40 mg/kg rat IgG (○), or 20 mg/kg human and 40 mg/kg rat IgG (◇) served as controls for IMC-18F1, MF1, or combination treatment, respectively. Points, mean ($n = 12$ per group) tumor volume; bars, SE.

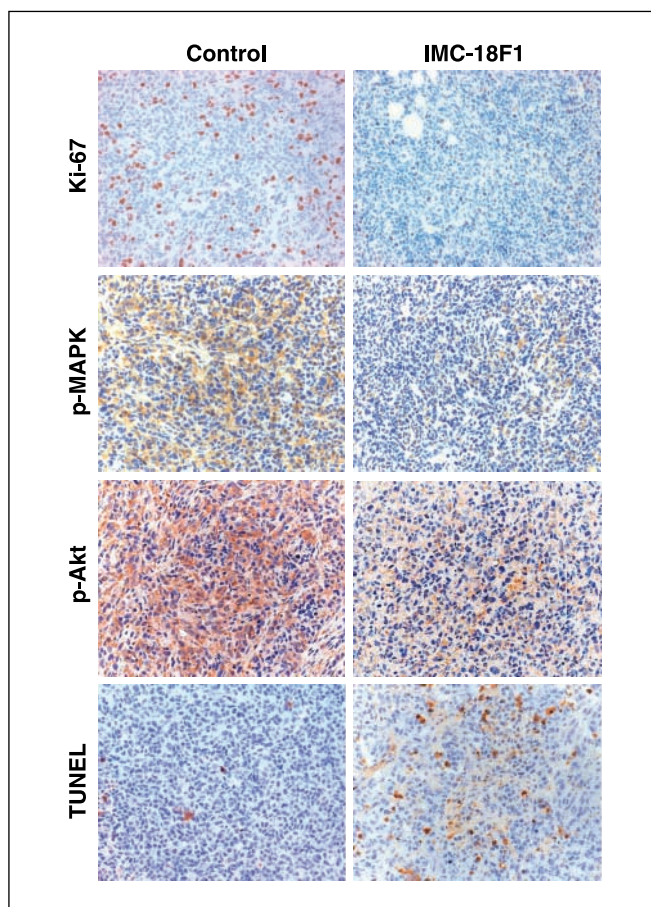


Fig. 7. Histology analysis of cell proliferation and intracellular activities of phosphorylated MAPK, phosphorylated Akt (*p-Akt*), and apoptosis in IMC-18F1-treated MDA-MB-231 xenograft tumors. Sections of the tumors were stained with antibodies to proliferation marker Ki-67, phosphorylated MAPK, and phosphorylated Akt and ApoptTag kits. A significantly reduced activity of proliferation, phosphorylated MAPK, and phosphorylated Akt and an increased apoptosis were found in IMC-18F1-treated tumor cells compared with the controls of human IgG-treated tumors. Representative tumor sections. Magnification, $\times 200$.

activity of IMC-18F1 plus MF1 in combination with chemotherapies in the MDA-MB-231 xenograft model. As shown in Fig. 8A-C and summarized in Table 2, adding treatment with IMC-18F1 (20 mg/kg) and MF1 (40 mg/kg) thrice weekly to chemotherapy with 125 mg/kg 5-FU and 62 mg/kg LV q7d, 3 mg/kg doxorubicin twice weekly, or 100 mg/kg cyclophosphamide q7d resulted in significantly increased antitumor efficacy ($P < 0.02$) compared with monotherapy with IMC-18F1 and MF1 or cytotoxic agent in the human breast tumor xenograft model.

Discussion

In this study, we report the development of a high-affinity human VEGFR-1 neutralizing mAb, IMC-18F1, and its antitumor activities in preclinical models. IMC-18F1 specifically binds to the extracellular domain of the human VEGFR-1 and effectively blocks binding of ligands VEGF-A, VEGF-B, and PlGF to VEGFR-1. We showed that IMC-18F1 blocked PlGF-induced VEGFR-1 autophosphorylation and activation of subsequent downstream MAPK signaling and arrested VEGF-A-stimulated

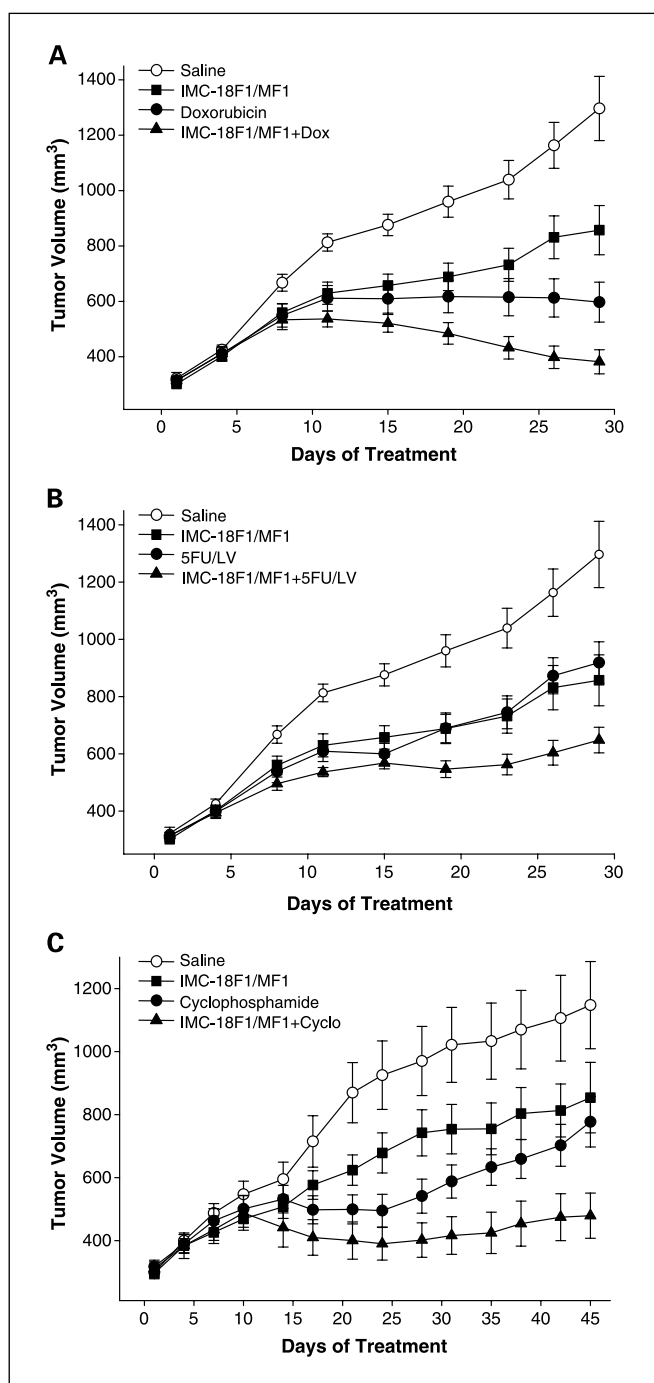


Fig. 8. Antitumor effects of IMC-18F1 and MF1 treatment in combination with cytotoxic agents on human breast tumor xenografts. Mice bearing established MDA-MB-231 tumor xenografts were treated with 20 mg/kg IMC-18F1 and 40 mg/kg MF1 thrice weekly (■), 3 mg/kg doxorubicin (*Dox*) twice weekly (A, ●), 125 mg/kg 5-FU/62 mg/kg LV q7d (B, ●), 100 mg/kg cyclophosphamide (*Cyclo*) q7d (C, ●), or IMC-18F1 and MF1 plus either cytotoxic agent (A-C, ▲). IMC-18F1 and MF1 treatment in combination with 5-FU/LV, cyclophosphamide, or doxorubicin resulted in a significantly enhanced antitumor efficacy ($P < 0.02$) compared with monotherapy with VEGFR-1 antibodies or any single cytotoxic agent. Statistical analysis of treatment outcomes is summarized in Table 2. Points, mean ($n = 12$ per group); bars, SE.

and PlGF-stimulated *in vitro* growth of breast carcinoma cells. These results show that IMC-18F1 is a potent inhibitor of intracellular activation of the VEGFR-1 and MAPK signaling for cell survival and growth, consequently resulting in preventing

the growth of human breast cancer cells *in vitro* and *in vivo* in xenograft models. Indeed, we have shown in xenograft studies that treatment of mice bearing established tumors with IMC-18F1 significantly suppressed the growth of human breast tumor xenografts. Consistent with the *in vitro* results, histologic analysis of the IMC-18F1-treated tumor xenografts indicated a decrease in MAPK and Akt activation and proliferative activity in tumor cells in addition to an increase in tumor cell apoptosis. IMC-18F1 has strict specificity with human VEGFR-1 and does not bind to mouse VEGFR-1; therefore, the antitumor effect of the antibody is primarily a result of direct inhibition of VEGFR-1 activation and subsequent downstream kinase signalings in cancer cells. These results have suggested that IMC-18F1-mediated blockade of VEGFR-1 activation is sufficient to suppress the growth of VEGFR-1-expressing breast tumors *in vivo*.

We and others have previously shown that treatment of mice with a mouse VEGFR-1-specific antibody MF1 significantly inhibits tumor angiogenesis, resulting in suppression of the growth of A431 epithelial tumor xenografts (13). VEGFR-1 antibody, when used in cancer patient, is anticipated to exert dual antitumor and antiangiogenic mechanisms of action through blockade of VEGFR-1-mediated functions in cancer cells and tumor vascular endothelium, respectively. To mimic the clinical scenario, we assessed antitumor activity of cotreatment with IMC-18F1 and the anti-mouse VEGFR-1 antibody MF1, which inhibits tumor neovascularization, in tumor xenograft models. As expected, the treatment with the dual anti-VEGFR-1 antibodies significantly increased inhibitory effects on the growth of tumor xenografts compared with treatment with either antibody alone. This result shows that treatment with VEGFR-1 antagonist antibody has a profound antitumor effect through inhibiting the VEGFR-1 activation-mediated cellular functions in both cancer and endothelial cells and possibly in other noncancer VEGFR-1-positive cells that contribute to neovascularization and tumor progression. Furthermore, we evaluated whether VEGFR-1 antibody treatment would potentiate antitumor efficacy of cytotoxic agents against breast tumor. Our xenograft studies showed that anti-human and mouse VEGFR-1 antibodies enhanced antitumor effect of chemotherapy with 5-FU/LV, cyclophosphamide, or doxorubicin. These results provide a foundation that the neutralizing anti-VEGFR-1 antibody has a therapeutic potential for monotherapy or combination treatment with chemotherapies in breast cancer and possibly other cancer indications.

A considerable number of studies have shown that VEGFR-1 plays an essential role in regulating pathologic angiogenesis in tumor, intraocular neovascular disorders, and other pathologic conditions (3-5), although a soluble form of VEGFR-1 was reported to play a role as a VEGF decoy for regulating VEGF signaling in vascular endothelial cells during vasculogenesis and early embryogenesis (30). Deficiency of VEGFR-1 tyrosine kinase domain resulted in decreased tumor vascularization and reduction of tumor growth in tumor models (31), suggesting the importance of VEGFR-1 kinase signaling in mediating tumor angiogenesis. In addition, blockade of VEGFR-1 activation by anti-VEGFR-1 antibody inhibited PlGF-induced angiogenesis and tumor neovascularization through preventing recruitment of bone marrow-derived endothelial and monocyte progenitor cells into tumor vascular endothelium (13, 32). Consistent with a previous report of VEGFR-1 tyrosine kinase

Table 2. Evaluation of IMC-18F1 and MF1 combined with cytotoxic agents compared with monotherapies against established MDA-MB-231 human breast tumor xenografts

Agent*	Dose (mg/kg)	Mean tumor volume (mm ³ ± SE)	%T/C [†] (d)	P (RM-ANOVA)
Control	NA	1296 ± 116	NA	
IMC-18F1 and MF1	20/40	857 ± 89	69 (29)	0.005 vs control
5-FU/LV	125/65	919 ± 73	73 (29)	0.008 vs control
IMC-18F1 and MF1 + 5-FU/LV	20/40 + 125/65	648 ± 45	51 (29)	<0.0001 vs control <0.02 vs antibodies [‡] 0.0004 vs 5-FU/LV
IMC-18F1 and MF1	20/40	857 ± 89	69 (29)	0.005 vs control
Doxorubicin	3	597 ± 72	54 (29)	<0.0001 vs control
IMC-18F1 and MF1 + doxorubicin	20/40 + 3	382 ± 43	30 (29)	<0.0001 vs control <0.0001 vs antibodies [‡] 0.01 vs doxorubicin
Control	NA	1,148 ± 138	NA	
IMC-18F1 and MF1	20/40	854 ± 112	74 (45)	0.1 vs control
Cyclophosphamide	100	777 ± 80	68 (45)	0.001 vs control
IMC-18F1/MF1 + cyclophosphamide	20/40 + 100	479 ± 72	42 (45)	<0.0001 vs control <0.0001 vs antibodies [‡] 0.005 vs cyclophosphamide

Abbreviations: RM-ANOVA, repeated measures ANOVA; NA, not available.

*IMC-18F1 (20 mg/kg) and MF1 (40 mg/kg) were administered i.p. thrice weekly. 5-FU/LV (125/62 mg/kg) and cyclophosphamide (100 mg/kg) were given i.p. once weekly. Doxorubicin (3 mg/kg) was given i.p. twice weekly.

[†]T/C, tumor growth inhibition, where T is the mean tumor volume of treated group and C is the mean tumor volume of control group on the designated day.

[‡]IMC-18F1 and MF1.

activity contributing to promotion of lung metastasis, we found that treatment with an anti-mouse VEGFR-1 antibody MF1 effectively impeded pulmonary metastasis of mouse 4T1 breast tumors in syngenic tumor model.⁵ VEGFR-1 activation was also shown to stimulate the activation and migration of monocytes and the induction of a series of inflammatory cytokines through activation of phosphatidylinositol 3-kinase kinase/Akt and extracellular signal-regulated kinase 1/2 pathways, implying an active role of VEGFR-1 in mediating pathogenic activities of monocytes and macrophages in inflammation (9, 33). Increased focal macrophage infiltration in breast cancer was reported to be associated with increased angiogenesis and reduced relapse-free and overall survival (34). A role of VEGFR-1 in regulating the activities of tumor-associated macrophages promoting angiogenesis and tumor progression remains to be determined.

Coexpression of VEGF-A and its cognate receptors has been frequently detected in hematologic malignant cells and solid tumor cells (17–26). Specifically, a role for VEGFR-1 in the pathogenesis of leukemia has been reported by several laboratories (reviewed in ref. 5). In a recent study, VEGFR-1 was reported to modulate the distribution of acute leukemia within the bone marrow and regulation of leukemia survival and migration into the peripheral circulation (19). Antibody-mediated inhibition of VEGFR-1 impeded the exit of acute lymphocytic leukemia cells from bone marrow and prolonged the survival of the inoculated mice and induced an increased apoptosis in leukemia cells. It has also been reported that VEGFR-1 is expressed in multiple myeloma and lymphoma cells and plays a functional role in promoting proliferation of these cancer cells (20, 21). Treatment of mice bearing human

B-cell lymphoma with anti-human VEGFR-1 antibody resulted in suppression of the lymphoma xenograft growth (20). Blockade of VEGFR-1 activation by a neutralizing antibody was recently shown to abrogate fetal stromal cell-dependent proliferation of primary multiple myeloma cells *in vitro* through inhibiting paracrine stimulation-induced VEGFR-1 activation in the cancer cells (21). VEGF-A was found to act as an autocrine growth factor to induce cancer cell invasion through activation of MAPK, phosphatidylinositol 3'-kinase, and Akt kinase signaling pathways in VEGFR-1-expressing breast cancer cell lines (22). Inhibition of VEGFR-1 signaling by a neutralizing antibody was shown to inhibit VEGFR-1-specific ligand-induced activation of the MAPK signaling in colon and pancreatic cancer cells, resulting in arrested growth and invasion of these cancer cells (23, 24). We have shown in a recent study that blockade of VEGFR-1 activation by an anti-VEGFR-1 antibody results in suppression of the growth of human breast tumor xenografts through inhibiting subsequent activation of MAPK and Akt signaling in cancer cells (25). Moreover, VEGFR-1 activation in cancer cells has been found to contribute to the survival and invasion of colon and pancreatic carcinoma cells through the VEGFR-1-mediated epithelial-mesenchymal transition (35, 36). In addition to these findings in preclinical studies, a long-term follow-up clinical study has shown that VEGFR-1 expression in breast cancer is significantly correlated with high risk of metastasis, relapse, and poor outcome in patient with node-negative tumors (37). Collectively, studies to date have provided considerable evidences that VEGFR-1 plays a crucial role in mediating functions of survival, growth, and invasion in solid and nonsolid cancer cells to promote tumorigenesis and tumor progression in addition to modulating tumor neovascularization in nontumor cells. Hence, VEGFR-1 has been considered an attractive therapeutic target for cancer treatment.

⁵ Y. Wu et al., unpublished data.

Several VEGFR-1 therapeutic antagonists have been developed for antitumor therapy. VEGFR-1-specific RNA inhibitor ribozyme was reported to have promising antitumor activity in inhibition of angiogenesis, tumor growth, and metastasis in solid tumor models (38). Tyrosine kinase inhibitor inhibiting VEGFR-1 activation is effective in inhibition of the growth of VEGFR-1-expressing multiple myelomas driven by ligand-induced autocrine stimulation (39). The VEGFR-1-specific peptide with ligand binding blocking activity was shown to prevent the growth and metastasis of VEGF-secreting tumor cells *in vivo* through inhibition of VEGFR-1-mediated endothelial cell migration and tube formation (40). We have shown in this report and in previous studies that anti-VEGFR-1 antagonist antibody has therapeutic efficacy against human tumor xenografts through inhibiting VEGFR-1 activation in cancer and endothelial cells.

Antibody-directed therapy targeting tumor growth-associated receptors is an effective approach to cancer because antibody treatment is target specific and has multiple effects on cancer cells. The receptor-specific antibody not only prevents ligand-stimulated activation of cognate receptors and subsequent downstream kinases signaling for survival, proliferation, and drug resistance in tumor cells but also induces receptor endocytosis and down-regulation (41) and mediates the immune effector responses of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity to attack cancer cells (42, 43). In addition, antibody-based therapies have shown a safer therapeutic profile owing to the nature of antibody comparing with RNA and peptide-based inhibitors. Several studies have shown that VEGFR-1 is present and functional in several solid tumor, lymphoma, multiple myeloma, and leukemia cancer cells (17–28). Our study reported here has laid the foundation for putting forward a novel VEGFR-1 antibody-based therapeutic strategy for monotherapy or combination treatment with chemotherapies in patients with a variety of cancer types. Further investigation into the antitumor activities of the VEGFR-1 antibody IMC-18F1 is warrant.

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