Effects of Anti-VEGF on Pharmacokinetics, Biodistribution, and Tumor Penetration of Trastuzumab in a Preclinical Breast Cancer Model

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Abstract

Both human epidermal growth factor receptor 2 (HER-2/neu) and VEGF overexpression correlate with aggressive phenotypes and decreased survival among breast cancer patients. Concordantly, the combination of trastuzumab (anti-HER2) with bevacizumab (anti-VEGF) has shown promising results in preclinical xenograft studies and in clinical trials. However, despite the known antiangiogenic mechanism of anti-VEGF antibodies, relatively little is known about their effects on the pharmacokinetics and tissue distribution of other antibodies. This study aimed to measure the disposition properties, with a particular emphasis on tumor uptake, of trastuzumab in the presence or absence of anti-VEGF. Radiolabeled trastuzumab was administered alone or in combination with an anti-VEGF antibody to mice bearing HER2-expressing KPL-4 breast cancer xenografts. Biodistribution, autoradiography, and single-photon emission computed tomography–X-ray computed tomography imaging all showed that anti-VEGF administration reduced accumulation of trastuzumab in tumors despite comparable blood exposures and similar distributions in most other tissues. A similar trend was also observed for an isotype-matched IgG with no affinity for HER2, showing reduced vascular permeability to macromolecules. Reduced tumor blood flow \( (P < 0.05) \) was observed following anti-VEGF treatment, with no significant differences in the other physiologic parameters measured despite immunohistochemical evidence of reduced vascular density. In conclusion, anti-VEGF preadministration decreased tumor uptake of trastuzumab, and this phenomenon was mechanistically attributed to reduced vascular permeability and blood perfusion. These findings may ultimately help inform dosing strategies to achieve improved clinical outcomes.

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Introduction

Despite advances in prevention, diagnosis, and treatment, cancer remains a major health challenge. In the past decade, significant progress has been made in the field of monoclonal antibody (mAb) therapy (1). However, the standard treatment option of single-drug therapy yields limited success due to low rates of complete remission and resistance. There is a growing consensus that the future of cancer treatment for solid tumors with immunotherapy lies in a combination therapy approach (1, 2).

Trastuzumab is a humanized mAb that specifically targets the extracellular domain of the human epidermal growth factor receptor-2 (HER2; refs. 3–5). The HER2 gene is amplified or the receptor overexpressed in approximately 15% to 25% of breast cancers (HER2-positive tumors; refs. 6, 7) and is associated with poor prognosis (8). Despite the success of trastuzumab treatment as a single agent (9) or in combination with chemotherapy (10), intrinsic and acquired resistance to trastuzumab treatment has led to the investigation of trastuzumab in combination with other therapeutic agents (11).

In breast cancer, HER2 and VEGF signaling pathways are linked, as upregulation of VEGF occurs in HER2-overexpressing breast cancer in the most aggressive cases (12). VEGF is responsible for recruiting de novo vascularization of the tumor, a process that is critical in all stages of
tumorigenesis (13–15). Consistently, bevacizumab (Avastin), a mAb specific for VEGFA (also referred to as VEGF) has shown efficacy against a variety of solid tumors (16). Anti-VEGF therapy has been evaluated in combination with chemotherapy in several tumor types, including metastatic breast cancer (17). A positive association was shown between HER-2/neu and VEGF expression (18), with HER2 expression levels linked to therapeutic response (19). Thus, combining antibodies specific for HER2 and VEGF could result in improved patient outcome compared with either agent alone. Indeed, the combination of bevacizumab and trastuzumab has shown partial tumor regression in xenograft models (20, 21). In a phase II study, bevacizumab combined with trastuzumab improved outcome in breast cancer patients with an overall response rate of 48% (22). Other promising strategies to facilitate antitumor activity include simultaneous targeting of HER2 and VEGF using a bi-specific antibody (23) or combining trastuzumab with an anti-VEGF fusion protein (24). At least 20 studies have been registered examining bevacizumab plus trastuzumab with cytotoxic chemotherapy and hormonal therapy in patients with HER2-positive breast cancer in neoadjuvant, adjuvant, and metastatic settings (ClinicalTrials.gov; accessed on June 30, 2011).

In xenograft models of human tumor growth, both human and mouse VEGF contribute to tumor angiogenesis. Trastuzumab alone has been shown to possess antiangiogenic properties through the downregulation of tumor produced VEGF (25, 26). Downregulation of VEGF signaling in tumors is pursued as an opportunity to restore vascularity to normalcy, a state requiring both spatial and temporal control of VEGF levels (27). However, it is not clear what effect suppressing tumor vascularization by bevacizumab will have on the distribution of trastuzumab to the tumor in vivo and resultant efficacy. In xenograft models, anti-VEGF therapy has induced rapid structural and morphologic changes in tumors (28), including reductions in both vascular permeability (29) and interstitial fluid pressure (30), but the cumulative effect of these changes on trastuzumab disposition to and within the tumor is not known. To address these questions, we evaluated the effect of a mouse-specific anti-VEGF antibody (B20-4.1), which also cross-reacts with human VEGF, on the tumor distribution and uptake of trastuzumab in a HER2-positive KPL-4 tumor xenograft model. In mice bearing human tumor xenografts, bevacizumab would only block tumor-derived human VEGF, whereas B20-4.1 blocks both tumor (human) and stroma-derived (murine) VEGF (31, 32). Trastuzumab or its isotype control (HER2 nonbinding antibody) were labeled with both 125I and 111In, allowing assessment of both real-time kinetics and cumulative tumor uptake, respectively (33).

Materials and Methods

Reagents and cell lines

Trastuzumab (Herceptin), anti-VEGF antibody (B20-4.1; refs. 31, 32), and an anti-herpes simplex virus glycoprotein D (anti-gD, designated henceforth as IgG) used as an isotype control for trastuzumab were obtained from Genentech, Inc. The HER2-expressing (3+) human breast cancer cell line KPL-4, obtained in 2006 from Dr. J. Kurebayashi (34), was used in most studies. The cells were cultured in RPMI 1640 media plus 1% L-glutamine with 10% FBS. The KPL-4 cell line was authenticated by short tandem repeat profiling (AMEL: X; CSF1PO: 11,13; D13S317: 12; D16S539: 12; D5S818: 11,13; D7S820: 9,10; TH01: 9; TPOX: 8; vWA: 14,19) and by single-nucleotide polymorphism genotyping. Both methods indicate that KPL-4 cells are unique when compared with a database of more than 1,000 human cancer cell lines.

A second model of human breast cancer, the transgenic line MMTV-HER2 Fo5, was also tested (35). The Fo5 model is a transgenic mouse model in which the human HER2 gene, under transcriptional regulation of the murine mammary tumor virus promoter (MMTV-HER2), is overexpressed in mammary epithelium. The overexpression causes spontaneous development of mammary tumors that overexpress the human HER2 receptor (35). The mammary tumor of one of these founder animals [founder #5 (Fo5)] has been propagated in subsequent generations of FVB mice by serial transplantation of tumor fragments. The model has been authenticated as HER2 overexpressing (2−3+) by HercepTest and by immunohistochemistry.

Radiochemistry

Iodine-125 was obtained as sodium iodide in 10−5 M sodium hydroxide from Perkin Elmer. Trastuzumab (75 μg) was labeled randomly through tyrosine residues at a specific activity of 11.3 μCi/μg with iodine-125 [125I] using the lodogen method (Pierce Chemical Co.; ref. 36). Radiosynthesis of 111In-trastuzumab (13.5 μCi/μg) was achieved through incubation of 111InCl3 and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated (randomly through lysines) mAb in 0.3 mol/L ammonium acetate pH 7 at 37°C for 1 hour. Purification of all radioimmunoconjugates was achieved using NAP5 columns equilibrated in PBS and confirmed by size-exclusion chromatography. In addition to radio purity and specific activity, immunoreactivities of the final radiolabeled products were tested (see below).

ELISA

ELISA allowed comparison of 125I- or DOTA-conjugated mAbs with the unmodified mAbs. Briefly, a fixed concentration of biotinylated trastuzumab (0.3 ng/mL) was mixed 1:1 with trastuzumab-DOTA or 125I-trastuzumab at varying concentrations (3 pg/mL-100 μg/mL) and captured on HER2 ECD (Genentech, Inc.) coated plates. Plates were developed using streptavidin–horse-radish peroxidase (Amersham Pharmacia Biotech) and tetramethyl benzidine substrate (Moss, Inc.). Kaleidograph version 4.03 (Synergy Software) was used to calculate IC50 values. For 125I-trastuzumab, a previously reported
total trastuzumab direct binding ELISA was used (37). ELISA of 125I- or DOTA-conjugated antibodies showed more than 80% recovery relative to unmodified antibodies.

**Tissue distribution and pharmacokinetic studies**

All animal studies were conducted in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the Genentech Institutional Animal Care and Use Committee. C.B-17 Icr SCID (severe combined immunodeficient; Inbred) female mice (Charles River Laboratories), weighing between 20 to 25 g were inoculated in the right mammary fat pad with approximately 3 million KPL-4 cells in a 50:50 suspension of Hanks' Buffered Salt Solution (Invitrogen) and Matrigel (BD Biosciences) in at most 0.2 mL/mouse. When mean tumor volume reached 200 to 300 mm³, mice received a single bolus injection via the tail vein containing 111In-trastuzumab (10 µCi) and 125I-trastuzumab (5 µCi), along with 0.1, 1.4, and 17 mg/kg unlabeled antibody. The rationale for carrying out pharmacokinetic and tissue distribution studies at 3 vastly different doses was not necessarily intended to mimic clinical exposure; instead, the goal was to study the biologic effects of anti-VEGF on trastuzumab uptake over a wide range of tumor receptor occupancies. To prevent thyroid sequestration of 125I, 100 µL of 30 mg/mL of sodium iodide was intraperitoneally administered 1 hour before dosing. A control group for trastuzumab was dosed with the mixture of 111In-IgG (10 µCi) and 125I-IgG (5 µCi) with the addition of unlabeled IgG to complete a total dose of 18 mg/kg. Select mice received anti-VEGF at a dose of 10 mg/kg 24 hours before dosing radiolabeled trastuzumab or its control mAb. The dose of anti-VEGF mAb was based on previously reported xenograft growth inhibition activity (16, 38), whereas the time interval was based on reported statistically significant reductions in vascular density of human xenografts in mice at 24 hours following anti-VEGF administration (28). Furthermore, a pharmacokinetic model simulation indicated that either a 5 mg/kg twice a week or 10 mg/kg weekly dosing regimen would result in a minimum trough concentration at steady state of 30 µg/mL, similar to that achieved in more than 90% of bevacizumab patients (38).

Terminal tissue harvest was done at 24, 48, 120, and 168 hours postinjection of trastuzumab or its control IgG (n = 3 per time point), and blood was collected via cardiac puncture under general anesthesia into lithium heparin Microtainers (BD Biosciences). Tissues collected consisted of heart, right kidney, lungs, spleen, muscle (gastrocnemius), normal mammary fat pad, and tumor. Tissue handling and analysis provided counts per minute values, which were used to calculate the percent of injected dose per gram of tissue (%ID/g) and area under the curve (AUC) as previously described (39-41). Note that %ID/g is a dose-normalized unit of concentration. Because animals were serially sacrificed with multiple blood samplings before takedown, we used a previously reported method (42) to calculate AUC₀₋₅ and the difference in AUC between groups with and without anti-VEGF treatment at each concentration, within each organ. P values were corrected for multiplicity within each organ using a step down procedure (43).

Distribution of 111In-trastuzumab (17 mg/kg), with or without anti-VEGF pretreatment, was also assessed in mice bearing MMTV-HER2 Fo5 tumors (200–250 mm³) transplanted into the mammary fat pad of Nu/Nu (nude) CRLL mice (Charles River Laboratories). Methods were analogous to those used in KPL-4 studies (see above).

**SPECT-CT imaging**

*In vivo* distribution was obtained by single-photon emission computed tomography/X-ray computed tomography (SPECT-CT) using modification of previously reported methods (39, 40). Mice (n = 1) received an average dose of 405 µCi (range: 383–416 µCi) of 111In-trastuzumab with or without anti-VEGF pretreatment as described above. Immediately after CT acquisition, SPECT images were acquired on two 20% windows centered at the 173- and 247-keV photopeaks of 111In using a high-resolution 5-pinhole collimator and a 5.5 cm radius of rotation. Mice were subsequently euthanized under sedation and tissues collected for gamma counting, in a manner identical to that used for the nonimaging arm of the study. SPECT quantitation was accomplished using Amira software (TGS).

**Autoradiographic imaging**

KPL4 tumor-bearing mice (n = 3) with and without anti-VEGF (as described above) were assessed by quantitative whole-body autoradiography. Each mouse received a single intravenous dose of 111In-trastuzumab plus unlabeled trastuzumab for a total dose of 17 mg/kg, 100 µCi/mouse. Animals were processed for whole-body cryosectioning, exposed and imaged as described previously (41).

**Mechanistic physiologic studies**

The vascular volumes, interstitial volumes, and rates of blood flow of KPL-4 tumors were measured *in vivo* by the indirect method for 99mTc red blood cell labeling (40), jugular cannula infusion of 111In-pentetate, and intravenous bolus injection of 82Rb chloride, respectively, as previously described (39).

**Immunohistochemistry**

Staining was done on 4-µm thick formalin-fixed paraffin-embedded tissue sections mounted on glass slides. Primary antibodies used were donkey antihuman IgG–biotin (Jackson ImmunoResearch), rabbit anti-parenchymal cell marker (clone Meca-32; Pharmingen), and rabbit anti-c-erbB-2 (HER-2; Dako). Detection was done with Vectastain ABC Elite reagent (Vector Labs) and Pierce metal enhanced DAB (Thermo Scientific).
Results

Radionuclide comparison

The blood profiles of both $^{125}$I and $^{111}$In isotopes were similar throughout the study, declining progressively over time (Fig. 1). Tumor uptake of $^{111}$In-trastuzumab at 0.1 mg/kg gradually increased before peaking at 38.7 ± 12.5%ID/g at 120 hours postdose (Fig. 2A) and remained at 30.7 ± 0.5%ID/g at 168 hours. Similarly, maximum tumor uptake of $^{125}$I-trastuzumab at 0.1 mg/kg (12.9 ± 8.0%ID/g) occurred 120 hours postdose and gradually decreased with time to 7.0 ± 2.1%ID/g at 168 hours postinjection. Two- to 3-fold higher uptake of $^{111}$In-trastuzumab compared with $^{125}$I-trastuzumab was generally observed at most time points and doses due to residualization (i.e., tumor cell retention) of radiometal (Fig. 2). In contrast, uptake of $^{111}$In-trastuzumab in most nonmalignant tissues was similar to that of $^{125}$I-trastuzumab at all doses (Fig. 3, Supplementary Table S1).

Antigen specificity and dose escalation

The dose-normalized concentration of radiolabeled trastuzumab was similar in blood (Fig. 1) and normal tissues (Fig. 3) at all doses of trastuzumab (0.1, 1.4, and 17 mg/kg). Similar levels were observed for radiolabeled IgG at 18 mg/kg. Tumor uptake of $^{111}$In-trastuzumab at all doses was higher than that of $^{111}$In-IgG at 17 to 18 mg/kg (Fig. 2).

An inverse relationship was observed between trastuzumab dose and tumor uptake of $^{111}$In- and $^{125}$I-trastuzumab (Fig. 3A and C). The highest tumor uptake was

Figure 1. Blood pharmacokinetic curves of $^{125}$I-labeled (A) and $^{111}$In-labeled (B) antibodies at various doses through 7 days. Solid data symbols correspond to control mice, whereas open symbols indicate mice treated with anti-VEGF at 24 hours before tracer administration.

Figure 2. Tumor pharmacokinetic curves with and without anti-VEGF (B20.4.1) pretreatment: trastuzumab at doses of 0.1 mg/kg (A), 1.4 mg/kg (B), 17 mg/kg (C), and a control IgG at a dose of 18 mg/kg (D).
observed at a dose of 0.1 to 1.4 mg/kg (38.7 ± 12.5 to 45.8 ± 4.4%/ID/g and 12.9 ± 8.0 to 10.9 ± 2.6%/ID/g for 111In- and 125I-trastuzumab, respectively). With the exception of spleen and liver, normal tissue uptake was not affected by trastuzumab dose. Uptake of 111In-IgG at 18 mg/kg was low and nearly constant, ranging from 7.2 ± 2.0 to 8.4 ± 0.5%/ID/g in tumor compared with 16.6 ± 2.0 to 26.3 ± 10.2%/ID/g for uptake of 111In-trastuzumab at 17 mg/kg (Fig. 2D). Relative to radiolabeled trastuzumab, much lower 120-hour tumor uptakes of only 8.2 ± 1.3 and 4.20 ± 0.03%/ID/g for 111In- and 125I-IgG, respectively, were detected (Fig. 3A and C).

Dose-normalized cumulative tumor uptake of 111In-trastuzumab at 0.1 mg/kg was 204 ± 5%/ID/g and was higher than that of 125I-trastuzumab (68.9 ± 3.5%/ID/g × d) (Supplementary Table S1). Dose escalation caused a significant decrease in measured AUCo.7 of radiolabeled trastuzumab, with a 25% to 30% lower dose-normalized cumulative tumor uptake of 125I- and 111In-trastuzumab, respectively, at 17 mg/kg (48.4 ± 4.1 and 153 ± 5%/ID/g × d) than at 0.1 mg/kg (68.9 ± 3.5 and 204 ± 5%/ID/g × d). In contrast, calculated AUCo.7 in the normal tissues showed approximately similar values at all doses.

**Anti-VEGF pretreatment**

As shown in Fig. 2A, non-pretreated mice at tracer dose (0.1 mg/kg) had 120-hour tumor uptakes of 38.7 ± 12.5 and 12.9 ± 8.0%/ID/g for 111In- and 125I-trastuzumab, respectively, whereas mice pretreated with anti-VEGF had 120-hour tumor uptakes of 18.0 ± 5.8 and 6.0 ± 4.0%/ID/g, respectively. Similarly, mice injected with an escalating dose of trastuzumab had reduced tumor accumulation at all time points when pretreated with anti-VEGF (Fig. 2A–C), whereas radioactivity levels were similar in normal tissues (Fig. 3). Accumulation of 111In- and 125I-IgG in tumor was reduced after pretreatment with anti-VEGF, as shown by the lower uptake that was achieved with the 18 mg/kg dose of IgG (Fig. 2D), whereas the uptake was similar in normal tissues (Fig. 3).

Anti-VEGF pretreatment resulted in lower tumor accumulation of tracer trastuzumab (0.1 mg/kg) relative to the non-pretreated group (204 vs. 95%/ID/g × d for 111In-trastuzumab, and 70 vs. 33%/ID/g × d for 125I-trastuzumab; Fig. 4, Supplementary Table S1). Similarly, with higher doses (1.4 and 17 mg/kg), the magnitude of tumor AUCo.7 diminishes, as previously seen at tracer dose, without altering the biodistribution of antibody in blood and normal tissues. Consistent with data from the KPL-4 tumor model herein, anti-VEGF pre- or posttreatment also reduced overall trastuzumab uptake into MMTV-HER2 Fo5 tumors (Supplementary Fig. S1).

**SPECT-CT imaging**

Forty-eight-hour SPECT-CT images of 111In-trastuzumab in KPL-4 tumor-bearing mice showed higher radio-tracer uptake in tumor with tracer antibody dose (1.4 mg/kg; Fig. 5A) compared with tumor uptake in animals receiving 14 mg/kg (Fig. 5B). Analogous to the nonimaging biodistribution results shown in Fig. 3A, a high codose of nonradioactive trastuzumab caused a reduction in tumor uptake of 111In-trastuzumab by...
SPECT-CT at 48 hours postinjection (31.5% and 18.5% in trastuzumab-only and anti-VEGF–treated mice, respectively) and showed evidence of specific accumulation of trastuzumab in tumors.

Forty-eight-hour SPECT-CT images of 111In-trastuzumab in KPL-4 tumor–bearing mice with anti-VEGF pretreatment showed a 58.2% and 50.2% reduction in tumors, at total trastuzumab doses of 1.4 mg/kg and 14 mg/kg, respectively, compared with animals receiving no anti-VEGF (Fig. 5A and B). Similar percent reductions (50%–60%) in tumor uptake were observed by SPECT-CT at 72 hours and showed excellent agreement (49%–51%) with corroborative terminal tissue distribution done by gamma counting immediately after noninvasive imaging (data not shown). Furthermore, these anti-VEGF–mediated reductions in tumor uptake are fairly consistent with the nonimaging biodistribution results shown in Fig. 2A and C, where at 48 hours, reductions in 111In-trastuzumab uptake of 61% and 56% were observed at 0.1 and 17 mg/kg, respectively.

### Autoradiographic imaging

Intratumoral localization of 111In-trastuzumab in KPL-4 tumor–bearing mice, as determined by quantitative autoradiography, is depicted in Fig. 5E and F. The left panel shows representative digital pictures of sagittal tumor sections 48 hours after administration of 111In-trastuzumab at 17 mg/kg, with and without anti-VEGF pretreatment. Autoradiographic imaging of these sections, right panel, showed a differential spatial distribution of 111In-trastuzumab as a result of anti-VEGF pretreatment. A more homogenous distribution of 111In-trastuzumab is observed in tumors receiving 111In-trastuzumab alone compared with that of anti-VEGF–pretreated tumors. In addition, a 44 ± 5% reduction of trastuzumab obtained from the autoradiogram analysis at the 48-hour time point is in agreement with the results obtained from SPECT-CT quantification and direct biodistribution measurements of the tumors.

### Mechanistic physiologic studies

The measured physiologic parameters for KPL-4 tumor and various tissues in the presence and absence of anti-VEGF are summarized in Supplementary Table S2. Among the 3 parameters measured, only blood flow (Q) in tumor showed a statistically significant difference between anti-VEGF treatment and lack thereof. Furthermore, no differences were detected in any nonmalignant...
tissue. The average rates of blood flow calculated for KPL-4 xenografts without (99 ± 28 μL/g/min) and with (49 ± 12 μL/g/min) anti-VEGF pretreatment were statistically different (P < 0.05) (Supplementary Table S2).

Immunohistochemistry

Decreased vasculature was observed by MECA-32 staining in tumors treated with anti-VEGF and trastuzumab compared with trastuzumab-only treatment (Fig. 6A and B). Remaining vasculature was present primarily at the periphery of tumor lobules. Bound trastuzumab (Fig. 6E and F) was predominately limited to the periphery of tumor lobules in anti-VEGF/trastuzumab-treated tumors, consistent with distribution of detected vascular elements in these tumors. HER2 antigen (Fig. 6C and D) was detected with slightly less intensity in anti-VEGF and trastuzumab-treated groups compared with trastuzumab-only–treated groups. The distribution of HER2 sites was variable in anti-VEGF/trastuzumab-treated tumors and not necessarily associated with peripheral vascular distribution.

Discussion

These studies represent the first direct comparison of trastuzumab disposition alone and in the presence of anti-VEGF therapy using a species cross-reactive anti-VEGF antibody (B20-4.1) in a xenograft tumor model in mice. The cross-reactive nature of the B20-4.1 antibody is of importance in that it displays immunoreactivity with both human VEGF (tumor derived) and murine (host) VEGF (stromal/vasculature derived; ref. 32), yielding an...
experimental system that is somewhat analogous to the clinical scenario. This approach enabled quantitative analysis of trastuzumab distribution to be assessed with both 125I- and 111In-radionuclides, in view of known differences in their biodistribution patterns when conjugated to antibodies (33). This study also showed the effects of preadministration of anti-VEGF on biodistribution and pharmacokinetics of radiolabeled trastuzumab. Furthermore, the results reaffirmed that suitably labeled trastuzumab can be used for noninvasive detection of bound trastuzumab in preclinical tumor models, with sufficient sensitivity to detect anti-VEGF-mediated changes in tumor pharmacokinetics.

Tumor uptake was superior for 111In-trastuzumab at all time points studied compared with that of 125I-trastuzumab. This is expected due to the residualizing properties of the 111In-DOTA label that is trapped intracellularly after internalization and proteolytic degradation of targeting antibody by tumor cells. Indium-111-trastuzumab accumulates at the target site, enabling the cumulative assessment of uptake over time (33). In contrast, trastuzumab labeled with nonresidualizing 125I showed lower retention of 125I-trastuzumab, allowing for the assessment of real-time kinetics of antibody distribution (33).

The effect of anti-VEGF on tumor uptake of trastuzumab was evaluated by coadministering 111In-DOTA- and 125I-labeled trastuzumab with or without pretreatment of anti-VEGF. Irrespective of the trastuzumab radioactive probe or dose, there was no anti-VEGF effect on the overall exposure of trastuzumab in the blood (Figs. 2 and 4). These data show that any change in trastuzumab uptake by tumors in the presence of anti-VEGF was not a consequence of altered exposure of trastuzumab but was rather a tumor-specific phenomenon.

As depicted in Fig. 1, preadministration with anti-VEGF resulted in decreased tumor uptake of the anti-HER2 antibody in the KPL-4 xenograft model and was not dependent on the dose of trastuzumab administered or the radiolabeling probe used. This effect of anti-VEGF was more pronounced when quantifying trastuzumab

Figure 6. Immunohistochemical staining of KPL-4 tumor sections for the MECA-32 vascular endothelium marker (brown; A, B), HER2 receptors (pink; C, D), and bound trastuzumab (blue; E, F). Images in A, C, and E correspond to trastuzumab (17 mg/kg) only treated mice, whereas those in B, D, and F are derived from mice receiving a single anti-VEGF dose (10 mg/kg) 24 hours before trastuzumab. Tumors were harvested at 24 hours after intravenous administration of trastuzumab. Scale bar, 500 μm. Insets display same tumor sections at a larger scale (2.0 mm).
accumulation by using $^{111}\text{In}$-DOTA. Furthermore, the effect of anti-VEGF on $^{111}\text{In}$-trastuzumab tumor uptake was less pronounced by day 7, possibly a consequence of diminished effects of the anti-VEGF antibody due to clearance. To show that these results were not unique to trastuzumab, tracer studies were carried out to compare uptake using an isotype control IgG. Interestingly, anti-VEGF also diminished tumor uptake of the control antibody (Fig. 1D). The decrease in tumor uptake of IgG confirms that at least one mechanism of action for anti-VEGF is to produce vascular permeability changes in the tumor, whereas that of most normal organs was unaffected (Figs. 3 and 4). This is an important observation, as it suggests that anti-VEGF treatment has the potential to reduce antibody distribution to the tumor independent of antibody specificity. Reduced tumor uptake of radiolabeled trastuzumab by anti-VEGF administered at 24 hours before or after the tracer was also observed in an additional tumor model (Fo5; Supplementary Fig. S1), indicating that the effect of anti-VEGF on tumor uptake of trastuzumab is independent of the dose schedule or high HER2-expressing tumor model used.

Although overall distribution to normal tissues was largely unaffected by anti-VEGF, it must be noted that at lower doses (0.1 and 1.4 mg/kg), trastuzumab retention in the spleen and liver was diminished in the presence of anti-VEGF (compare Fig. 3A and B, and Fig. 3C and D). This is likely a consequence of nonlabeled antibody competition for binding to FcγR due to extremely low endogenous IgG levels in SCID mice (44), as this effect was not seen when greater nonlabeled antibody concentrations were administered (17 mg/kg trastuzumab or 10 mg/kg anti-VEGF). The kidney presented with a trend for greater uptake of $^{111}\text{In}$-antibody (Fig. 3A and B, and Supplementary Table S1) despite treatment and isotype, which is consistent with the renal tubular reabsorption of $^{111}\text{In}$-DOTA degradation products (45). In normal tissues, distribution of trastuzumab and an isotype control did not distinguish from each other, again consistent with the lack of HER2 expression in these tissues.

The acute effect of anti-VEGF on trastuzumab tumor uptake observed by the biodistribution study described above was confirmed by SPECT-CT. At a low dose of $^{111}\text{In}$-trastuzumab, anti-VEGF administration 24 hours before trastuzumab caused a 58.2% reduction in tumor uptake relative to untreated (Fig. 5A and C). At the 14 mg/kg dose of $^{111}\text{In}$-trastuzumab, anti-VEGF led to a 50.3% reduction in tumor uptake (Fig. 5B and D). Consistently, by autoradiography, a 42% reduction in tumor uptake of a 17 mg/kg $^{111}\text{In}$-trastuzumab dose was observed in the presence of anti-VEGF (Fig. 5E and F).

Tumor blood perfusion, as measured by radiorubidium uptake and expressed as a function of cardiac output, had a baseline value of 98.9 ± 28.0 µL/g/min in the absence of anti-VEGF (Supplementary Table S2). However, at 24 hours after treatment with anti-VEGF, the rate of blood flow had significantly ($P < 0.05$) decreased to 48.3 ± 12.1 µL/g/min (Supplementary Table S2). This effect was tumor specific, as anti-VEGF did not alter blood flow to nonmalignant tissues, and these findings agree with our previous data (39). The rubidium radionuclide $^{86}\text{Rb}$ is an ideal marker for regional blood flow in tumors and other tissues, because its small size (roughly 1.5 Å) dictates that its entry into tissues in a brief (<1.5 min) time period should be dominated by blood flow and thus minimally permeability limited (46). Such changes in tumor blood flow dynamics may be related to nitric oxide (NO) levels (47), as inhibition of NO production is one of the documented consequences of VEGF inhibition (48). Indeed, our findings are consistent with a previous clinical study using bevacizumab, in which observed reductions in the volume transfer constant ($K_{\text{trans}}$) by dynamic contrast-enhanced MRI (DCE-MRI) were consistent with decreased tumor blood flow and/or reduction in vessel permeability (28). $K_{\text{trans}}$ is a composite estimate of both the blood flow and the permeability surface area product per unit volume of tissue for transendothelial transport between plasma and extravascular space.

Although a reduction in relative blood volume of tumors following treatment with a different cross-reactive anti-VEGF antibody was previously reported (28), we observed no anti-VEGF–induced change in tumor blood volume (Supplementary Table S2). This could be related to the method of measurement, as blood volumes were assessed by noninvasive DCE ultrasound imaging of microbubbles (1–3 µm) in the previous work (28) and by detection of radiolabeled murine red blood cells (6 µm) following invasive tumor harvest in this work. Despite the apparent discrepancy in anti-VEGF effects on tumor blood volume, reductions in tumor vascular density were observed by immunohistochemistry in both the previous (28) and current studies (Fig. 6A and B). These seemingly contradictory findings raise the possibility that anti-VEGF may achieve tumor vessel normalization (27), in part, by pruning nonfunctional blood vessels carrying little to no blood.

Anti-VEGF decreased vasculature density and limited the distribution of trastuzumab to the periphery of tumor lobules. Representative tumor sections are shown in Fig. 6, revealing that anti-VEGF not only reduced tumor uptake of trastuzumab but also restricted its distribution to the periphery of the tumor (Fig. 6E and F). Decreased vascular staining in anti-VEGF pretreated tumors is obvious at the periphery of neoplastic lobules, especially in the interior of mass compared with mice receiving trastuzumab only (Fig. 6A and B). It seems reasonable to speculate from these ex vivo visualizations that the relatively decreased tumor vasculature may regulate, at least in part, the rate of macromolecule extravasation across tumor blood vessels long known to be associated with the qualitatively poor penetration into the tumors (49). These results were corroborated by both autoradiography and immunohistochemical staining observed in Figs. 4F and 5F, respectively. Moderate to high expression of HER2
was evenly distributed throughout the tumor mass in mice receiving trastuzumab-only (4+1) and anti-VEGF followed by trastuzumab (3+- Fig. 6C and D), respectively. The slightly weaker staining of HER2 in anti-VEGF-treated tumors raises the possibility that down-regulation of HER2 expression by anti-VEGF may be at least partially responsible for the decrease in trastuzumab tumor uptake. Despite the results herein, considerable preclinical evidence for efficacy of trastuzumab in combination with bevacizumab must still be weighed (20, 21). Furthermore, a phase II trial evaluating combination of bevacizumab and trastuzumab in first-line treatment of 50 patients with HER2-positive advanced breast cancer reported encouraging results (50). Nevertheless, further investigations are necessary to better understand the complex interplay between each constituent of the combination and the resulting effects on overall patient benefit. In conclusion, these results show that (i) tumor uptake of trastuzumab decreased with concomitant administration of an anti-VEGF antibody; (ii) blood pharmacokinetics were not impacted by anti-VEGF administration, indicating that the differences in tumor uptake were not blood exposure driven; (iii) the impact of an anti-VEGF antibody on the tumor uptake of a postadministered antibody was independent of antibody specificity and tumor type; (iv) the effect of anti-VEGF was restricted to tumors as most normal tissues remained unaffected; and (v) mechanistic studies suggest that reductions in both tumor blood flow and vascular permeability to macromolecules contribute to the observed changes in tumor pharmacokinetics of antibodies.

Disclosure of Potential Conflicts of Interest
All authors have held financial interest as employees of Hoffmann-La Roche or Genentech, a member of the Roche Group. Herceptin (trastuzumab) is marketed in the U.S. by Genentech and internationally by Roche.

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