Temporal Modulation of HER2 Membrane Availability Increases Pertuzumab Uptake and Pretargeted Molecular Imaging of Gastric Tumors

Running title: Modulating HER2 for Pretargeted Imaging

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Word Count: 5,023

Financial disclosure: This research was funded in part through the NIH/NCI Cancer Center Support Grant P30 CA008748, NIH U01 CA221046, NIH R01 CA204167, the MSK Geoffrey Beene Cancer Research Center, a Tow Foundation Postdoctoral
Fellowship from the MSK Center for Molecular Imaging and Nanotechnology (P.M.R.P.), and a Ruth L. Kirschstein National Research Service Award postdoctoral fellowship (L.M.C. NIH F32-EB025050).

**Disclosure of potential conflicts of interest:** In regards to this publication, Jason S. Lewis has received research reagents from Genentech and Y. Janjigian has received research funding from Genentech/Roche.

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2) is used as a tumor biomarker and therapeutic target. Pertuzumab is an anti-HER2 antibody, and its binding to tumor cells requires HER2 to be present at the cell membrane. However, the cellular distribution of HER2 protein in gastric tumors is dynamic, and HER2 internalization decreases antibody binding to tumor cells. These features preclude the use of pretargeted strategies for molecular imaging and therapy. We explored the modulation of HER2 endocytosis as a strategy to improve pertuzumab uptake in HER2-positive gastric tumors and allow the use of a pretargeted imaging approach.

Methods: We conducted in vitro and in vivo studies with NCI-N87 gastric cancer cells to determine how HER2 endocytosis affects pertuzumab binding to tumor cells. Lovastatin, a clinically approved cholesterol-lowering drug, was used to modulate caveolae-mediated HER2 endocytosis.

Results: Administration of lovastatin to NCI-N87 cancer cells resulted in significant accumulation of non-activated HER2 dimers at the cell surface. Pretreatment of NCI-N87 cells with lovastatin increased in vitro specific accumulation of membrane-bound zirconium-89 ($^{89}$Zr)-labeled pertuzumab. Lovastatin-enhanced pertuzumab tumor uptake was also observed in NCI-N87 gastric cancer xenografts, allowing tumor detection as early as 4 hours and high-contrast images at 48 hours post-tracer administration via Positron Emission Tomography (PET). Temporal enhancement of HER2 membrane availability by lovastatin allowed imaging of cell-surface HER2 with trans-cyclooctene (TCO)-conjugated antibodies and fluorine-18 ($^{18}$F)-labeled tetrazine (Tz).
Conclusions: Temporal pharmacological modulation of membrane HER2 may be clinically relevant and exploitable for pretargeted molecular imaging and therapy in gastric tumors.

Keywords: HER2, pertuzumab, gastric tumors, lovastatin, pretargeting
INTRODUCTION

Members of the human epidermal growth factor receptor (HER) family (HER1, HER2, HER3, and HER4) are membrane receptor tyrosine kinases that in response to extracellular signals trigger downstream oncogenic signaling cascades [1]. Aberrant cancer signaling – in pathways activated by HER family members – results from dysregulated receptor activation (mediated by receptor homo- and heterodimerization upon ligand binding, for example), receptor overexpression, or specific mutations [1-4]. HER2 appears to have no direct ligand, and its indirect activation most likely is triggered by dimerization with other members of the HER family [1, 5]. Many cancers have amplification of the \textit{HER2} gene and/or overexpression of HER2 protein [6, 7]. Therapies targeting HER2 have been very successful in the treatment of breast cancer [8, 9], and monoclonal antibodies (trastuzumab and pertuzumab), antibody-drug conjugates (ado-trastuzumab emtansine), and tyrosine kinase inhibitors targeting both HER1 and HER2 (lapatinib) are clinically approved for the treatment of breast cancer.

HER2 is also a clinical biomarker and therapeutic target in patients with gastric tumors [3, 10-16]. Indeed, treating patients with HER2-positive metastatic gastric or gastro-oesophageal junction tumors with HER2-targeting trastuzumab plus chemotherapy has yielded improved overall survival compared to chemotherapy alone [10]. Based on data supporting a synergetic effect of trastuzumab and pertuzumab [17], a dual HER2 blockade plus chemotherapy approach was tested in the JACOB trial. However, this combination did not significantly improve overall survival in patients with HER2-
positive metastatic gastric or gastro-oesophageal junction cancer compared with placebo [18]. Notably, a current limitation is that selection of patients for HER2-targeted trials is largely based on the assessment of HER2 status through immunohistochemistry of tumor biopsy specimens. This approach incompletely captures the cellular dynamics of HER2 and its heterogeneous expression in gastric tumors [15]. The use of molecular imaging to evaluate the expression of receptors of the HER family is a promising strategy to improve patient selection for anti-HER therapies and monitoring therapeutic response [19-23].

HER2 antibodies (trastuzumab or pertuzumab) radiolabeled with zirconium-89 ($^{89}$Zr) have the potential to target and image HER2-positive tumors [21-24]. However, clinical studies have reported that $^{89}$Zr-labeled antibodies do not always accumulate in HER2-positive tumors [25]. Immunohistochemical staining of gastric tumors reveals non-uniform membrane expression of HER2 [15], which contributes to low accumulation of antibodies in these tumors [18, 26, 27]. Moreover, endocytic trafficking mediates HER2 internalization and further reduces the availability of HER2 at the cell membrane, preventing binding with antibodies such as trastuzumab and pertuzumab and dampening their therapeutic efficacy [27-30].

The internalization of HER2 to the intracellular compartment not only decreases the ability of $^{89}$Zr-labeled antibodies to target HER2-positive tumors, but also precludes the use of pretargeted strategies for molecular imaging and therapy [31-33]. Pretargeting approaches have been developed to reduce radiation doses to healthy tissues associated with the use of antibodies radiolabeled with long-lived radionuclides. The inverse
electron demand Diels-Alder click chemistry-based \textit{in vivo} pretargeting approach involves: 1) injection of a tumor-targeting antibody bearing a clickable handle, 2) accumulation of the antibody in tumor(s) over a period of 24-72 hours accompanied with clearance from the blood, 3) injection of a pharmacokinetically short-lived radioligand containing a clickable counterpart, and 4) \textit{in vivo} click between the radioligand and the membrane accumulated antibody [31, 32, 34]. Currently, the usefulness of such a pretargeted strategy for a rapidly internalizing antigen, such as HER2, is limited; antibody-mediated internalization of HER2 reduces the availability of the antibody and its associated clickable sites on the tumor for the incoming radioligand, which can bear an imaging or therapeutic radionuclide. HER2 is also a circulating antigen and the injected antibody will not only target the antigen-expressing tumor tissue, but it will also bind HER2 present in circulation. Therefore, in a pretargeted strategy, the small molecule radiotracer will react with antibody bound HER2 in circulation and increase the background-to-tumor ratios.

Caveolin-1 (CAV1), a protein present in cholesterol-rich structures at the cell membrane, mediates HER2 internalization and reduces HER2 availability at the cell membrane for binding with trastuzumab [27, 35, 36]. We have previously shown that temporal modulation of CAV1 protein with the cholesterol-lowering drug lovastatin improved HER2 stability at the cell membrane and increased and accelerated tumoral uptake of \textsuperscript{89}Zr-labeled trastuzumab.
In this study, we explored the hypothesis that modulation of HER2 endocytosis would improve the accumulation of pertuzumab in HER2-positive gastric tumors and allow the use of a pretargeted approach in HER2-positive tumors.

MATERIALS AND METHODS

Cell lines and treatments

The HER2-positive gastric/CAV1-positive NCI-N87 and HER2-negative/CAV1-positive breast MDA-MB-231 human cancer cell lines were purchased from American Type Culture Collection (ATCC, CRL-5822 and HTB-26) in 2014 and authenticated by the Memorial Sloan-Kettering Cancer Center (MSK) Integrated Genomics Operation Core using short tandem repeat analysis. NCI-N87 and MDA-MB-231 cells were used within 15 passages and were confirmed to be mycoplasma free. NCI-N87 cells were maintained at 37 °C in a humidified atmosphere at 5% CO₂ in RPMI-1640 growth medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 100 units/mL of both penicillin and streptomycin. MDA-MB-231 cells were cultured in ATCC-formulated Leibovitz's L-15 medium supplemented with 10% fetal bovine serum.

NCI-N87 cells were incubated with 25 μM of the active form of lovastatin (Millipore) in medium for 4 hours (h) prior to addition of pertuzumab. Control experiments were
performed by incubating NCI-N87 cancer cells in medium for 4 h before addition of pertuzumab.

**Western blot analysis**

Whole-protein extracts from control or lovastatin-treated NCI-N87 cells were obtained by cell scraping at 4 °C in radioimmunoprecipitation assay buffer [RIPA buffer: 150 mM sodium chloride (NaCl), 50 mM Tris hydrochloride (Tris-HCl), pH 7.5, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 2 mM phenylmethanesulfonyl (PMSF), 2 mM iodoacetamide (IAD), and 1x protease inhibitor cocktail (Roche, C852A33)]. After centrifugation at 16,000 x g for 10 minutes (min) at 4 °C, supernatants were used for protein quantification with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) and denatured with Laemmli buffer. Following electrophoresis and transfer to nitrocellulose membranes (Thermo Fisher Scientific, IB23001), the blots were incubated in 5% (m/v) BSA in Tris-buffered saline buffer-Tween (TBS-T, Cell Signaling Technology, 9997S), and probed with mouse anti-β-actin 1:20,000 (Sigma, A1978), rabbit anti-HER2 1:800 (Abcam, ab131490), rabbit anti-EGFR 1:800 (Abcam, ab52894), 1:500 rabbit anti-phospho-p44/42 MAPK (Cell Signaling, 9101S), 1:800 rabbit anti-p44/42 MAPK (Cell Signaling, 9102S), and 1:500 mouse anti-phosphotyrosine antibodies (EMD Millipore, 05-321X). After washing, the membranes were incubated with IRDye®800CW anti-Rabbit (925-32211) or anti-Mouse (925-32210) IgG 1:15,000 (LI-COR Biosciences) and imaged on the Odyssey Infrared Imaging System (LI-COR Biosciences) followed by densitometric analysis using ImageJ software.
**Immunoprecipitation assays**

For immunoprecipitation experiments, total cellular protein (500 μL of RIPA buffer containing 200 μg protein) was incubated with 10 μg of primary antibody Neu (F-11) agarose conjugate (Santa Cruz Biotechnology, sc-7301 AC) overnight at 4 °C with gentle rotation. The pellet (containing the immunoprecipitated fraction) was collected by centrifugation at 1,000 x g for 30 seconds (sec) at 4 °C and washed three times with RIPA buffer before resuspension in Laemmli buffer.

**Pertuzumab DFO and TCO conjugation**

Clinical-grade pertuzumab (Perjeta; Genentech) was conjugated with p-isothiocyanatobenzyl-desferrioxamine (DFO-Bz-NCS) or trans-cyclooctene (TCO) as described previously [22]. Briefly, a solution of pertuzumab (2.61 mg, 3.26 mg/mL) in PBS, pH 7.4 was adjusted to pH 8.4 with 1 M sodium bicarbonate solution (NaHCO₃). Thirteen molar equivalents of the bifunctional chelate DFO-Bz-NCS (Macrocyclics, Inc, 10 mg/mL, 13.3 mM) in dimethyl sulfoxide (DMSO) were added. For conjugation with TCO, pertuzumab (3.40 mg, 3.40 mg/mL) in PBS (pH 7.4) was adjusted to pH 8.5 using 0.1 M Na₂CO₃. Pertuzumab was then reacted with thirty molar equivalents of TCO-N-hydroxysuccinimide (NHS) (25.0 mg/mL, 94 mM prepared in N,N-dimethylformamide). The DFO or TCO conjugation reactions were prepared fresh before use by incubating the antibody with DFO-Bz-NCS or TCO-N-NHS at 37 °C for 90 min before purification with a PD10 desalting column (GE Healthcare). Antibody-DFO conjugate was used for radiolabeling with zirconium-89 and antibody-TCO conjugates were used for pretargeted strategies.
Radiolabeling of pertuzumab and tetrazines

Zirconium-89 was produced via proton bombardment of yttrium foil and isolated with high purity as $[^{89}\text{Zr}]\text{Zr}$-oxalate at MSK using previously reported procedures [37]. A neutralized solution of $^{89}\text{Zr}$-oxalate (37 MBq, pH 7.0–7.2) was added to pertuzumab-DFO (300 μg) in PBS at 37°C for 1 h before sequential purification with a Sephadex G-25 (PD10; GE Healthcare) desalting column and 50 kDa molecular weight cutoff regenerated cellulose centrifugal filter (Amicon; Millipore). $[^{89}\text{Zr}]\text{Zr}$-pertuzumab with radiochemical purity (RCP) ≥95% as determined by instant thin-layer chromatography was used for in vitro and in vivo studies.

No-carrier-added $[^{18}\text{F}]$fluoride was obtained via the $^{18}\text{O}(p,n)^{18}\text{F}$ nuclear reaction of 11-MeV protons on an $^{18}\text{O}$-enriched water target. $[^{18}\text{F}]\text{AlF-NOTA-PEG}_{11}$-Tz was synthesized following procedures described previously [32] and used with the same RCP specification (RCP>98% and MA=1.5 mCi/nmol) as determined by radio-high performance liquid chromatography (Supplementary Fig. 1).

Internalization assays and saturation-binding assays

For the internalization assays with $[^{89}\text{Zr}]\text{Zr}$-DFO-pertuzumab, control or lovastatin-treated cells were incubated with cell culture medium in the presence of 1 μM $[^{89}\text{Zr}]\text{Zr}$-DFO-pertuzumab for 90 min at 37 °C. Media containing non-cell-bound radiotracer was removed, and the cells were washed twice with PBS. Cell surface-bound radiotracer was collected by incubation at 4 °C for 5 min in 0.2 M glycine buffer containing 0.15 M NaCl.
and 4 M urea at pH 2.5. The internalized fraction was obtained after cell lysis with 1 M sodium hydroxide (NaOH). The three fractions were measured for radioactivity on a gamma counter calibrated for \(^{89}\text{Zr}\).

For the saturation-binding assays, cells were incubated with \(^{89}\text{Zr}\)-labeled pertuzumab (0–256 nM) in PBS (pH 7.5) containing 1% (m/v) human serum albumin (Sigma) and 1% (m/v) sodium azide (Acros Organics) for 2 h at 4 °C. Unbound radioactivity was removed and cells were washed three times with PBS. The cells were solubilized in 100 mM NaOH, recovered, and the total cell-bound radioactivity was measured on a gamma counter calibrated for \(^{89}\text{Zr}\). Total binding was plotted vs. the concentration of \(^{89}\text{Zr}\)-pertuzumab; the data were fit via non-linear regression with a one-site binding model in GraphPad Prism 7.00 to determine \(B_{\text{max}}\). The non-specific component was subtracted from the total binding to generate specific binding curves.

**In vitro blocking experiments**

Blocking experiments were performed by incubating cells with \(^{89}\text{Zr}\)-labeled pertuzumab in the presence of a 30-fold excess of trastuzumab or pertuzumab.

**Tumor xenografts**

Experiments in animals were conducted according to the guidelines approved by the Research Animal Resource Center and Institutional Animal Care and Use Committee at MSK. The first author (Pereira) has a Category C accreditation for animal research from the Federation of European Laboratory Animal Science (FELASA). We adhere to the Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines and to the
guidelines for the welfare and use of animals in cancer research. Eight- to 10-week old \textit{nu/nu} female mice (Charles River Laboratories) were injected subcutaneously on the right shoulder with 5 million NCI-N87 cells in a 150 μL cell suspension of a 1:1 (v/v) mixture of medium with reconstituted basement membrane (BD Matrigel, BD Biosciences). MDA-MB-231 cells were implanted orthotopically (5 million cells) in the lower right mammary fat pad in 50 μL of 1:1 Matrigel (BD Biosciences). The mice were housed in type II polycarbonate cages, fed a sterilized standard laboratory diet, and received sterile water \textit{ad libitum}. The animals were housed at approximately 22 °C, 60% relative humidity, and a 12 h light, 12 h dark cycle was maintained. After arrival, all mice were allowed to acclimate to the facility’s laboratory conditions for one week prior to experimentation.

The tumor volume ($V$, mm$^3$) was estimated by external vernier caliper measurements of the longest axis, $a$ (mm), and the axis perpendicular to the longest axis, $b$ (mm). The tumors were assumed to be spheroidal, and the volume was calculated using the equation $V = \frac{4\pi}{3} \times \frac{a}{2}^2 \times \frac{b}{2}$. When the volume of xenografts reached approximately 100 mm$^3$, mice were randomized into groups and treatments were initiated ($n = 5$ mice per group for biodistribution and $n = 3$ mice per group for PET imaging).

For the imaging experiments with $^{89}$Zr-labeled pertuzumab, mice were divided into two groups: \textit{Group I}, which received PBS orally 12 h prior to and at the same time as the tail vein injection of $[^{89}\text{Zr}]\text{Zr-DFO-pertuzumab}$ (4.44-5.18 MBq, 42-49 μg protein); and
Group II, which received lovastatin orally (8.3 mg/kg of mice) 12 h prior to and at the same time as the tail vein injection of $[^{89}\text{Zr}]\text{Zr-DFO-pertuzumab}$.

**In vivo blocking experiments**

Blocking experiments were performed in tumor-bearing mice injected with $^{89}\text{Zr}$-labeled pertuzumab in the presence of a 40-fold excess of trastuzumab or pertuzumab.

**Pretargeting**

For the pretargeting experiments, lovastatin (8.3 mg/kg of mice) was orally administered 12 h prior to and at the same time as the tail vein injection of pertuzumab-TCO or trastuzumab-TCO (0.42 nmol). At 24 h post-injection of antibody, $[^{18}\text{F}]\text{AlF-NOTA-PEG}_{11}\text{-Tz}$ (14.73-16.54 MBq, 0.83-1.01 nmol) was injected via the tail vein.

**Acute biodistribution studies**

Acute biodistribution studies were carried out according to previously reported methods [32].

**PET and PET/CT imaging**

Imaging experiments were conducted on a microPET Focus 120 scanner (Concorde Microsystems) or an Inveon PET/CT scanner (Siemens). Mice were anesthetized by inhalation of 1.5-2% isoflurane (Baxter Healthcare) in an oxygen gas mixture 10 min before recording PET images. PET data for each group ($n = 3$) was recorded, with mice under isoflurane anesthesia (1.5-2%), in list mode at 4, 8, 24, and 48 h after intravenous
injection of $[^{89}\text{Zr}]$Zr-DFO-pertuzumab. PET/CT data for each group ($n = 3$) was acquired 0.5, 1.5 and 4 h after injection of $^{18}$F-labeled Tz. List mode emission data were sorted into 2-dimensional (D) sinograms via Fourier rebinning; data were normalized to correct for non-uniform detector response, dead time count losses, and positron branching ratio, but no attenuation, scatter, or partial-volume averaging corrections were applied. A 3D ordered subset expectation maximization/maximum a posteriori (OSEM3D/MAP; 2 OSEM iterations/18 MAP iterations) was used for reconstruction, and each reconstructed image was smoothed by convolution with a 1.5-mm full width at half maximum (FWHM) Gaussian filter kernel to reduce noise. All images were visualized in AMIDE 1.0.4 software (http://amide.sourceforge.net).

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM). Groups were compared using the Student’s $t$ test.
RESULTS

Lovastatin increases HER2 dimerization without altering HER2 phosphorylation or activation

Temporal modulation of CAV1 with the cholesterol-lowering drug lovastatin increases HER2 availability at the cell membrane to enhance the binding of trastuzumab to gastric cancer cells [27]. Our previous work showed an increase in HER2 half-life at the cell membrane of NCI-N87 gastric cancer cells. Based on these findings, we investigated HER2 dimerization upon treatment of these cells with lovastatin. Homodimerization and heterodimerization of HER2 are known to lead to autophosphorylation and further induction of downstream pro-oncogenic signaling pathways [4, 5, 38]. Treatment of NCI-N87 gastric cancer cells with 25 μM of the active form of lovastatin for 4 h significantly enhanced the formation of HER2-HER2 homodimers (1.7 ± 0.3, n = 3) and HER2-EGFR heterodimers (2.0 ± 0.3, n = 3) in NCI-N87 gastric cancer cells (Fig. 1A). We did not detect HER2-HER3 heterodimers (data not shown), possibly due to the low levels of HER3 in NCI-N87 gastric cancer cells [17] or due to the fact that our experiments were not performed in the presence of a ligand [39]. Although lovastatin treatment increased HER2-HER2 and HER2-EGFR dimers, we did not detect alterations in HER2 phosphorylation or in phosphotyrosine-containing proteins (Fig. 1B). Additional Western blot analyses revealed that lovastatin treatment did not impact the downstream mitogen-activated protein kinase (MAPK) pathway (Fig. 1B). Collectively, our results are consistent with an increase in HER2 availability at the cell membrane upon treatment with lovastatin and the formation of non-activated dimerized HER2 receptors.
Lovastatin improves molecular imaging with $^{89}$Zr-labeled pertuzumab

Prompted by our previous studies demonstrating that lovastatin treatment increases the avidity of HER2-positive tumors for trastuzumab [27], we performed in vitro and in vivo studies with $^{89}$Zr-labeled pertuzumab. Pertuzumab, a HER2 heterodimerization inhibitor, binds to the dimerization hairpin on the extracellular domain II of HER2 [40]. Given our in vitro findings (Fig. 1A), we expected that changes in non-activated membrane HER2 dimers upon treatment with lovastatin would affect the ability of pertuzumab to bind tumor cells. Cellular fractionation experiments revealed a significant increase in membrane-associated $^{89}$Zr-labeled pertuzumab in cells pretreated with lovastatin compared to the control group (Fig. 2A). The increase in membrane-bound pertuzumab was accompanied by a significant decrease in the amount of internalized radioactivity (Fig. 2A). Additional competitive radioligand saturation-binding assays confirmed that lovastatin treatment increases pertuzumab binding to membrane HER2 in NCI-N87 cells ($B_{\text{max}}$; Supplementary Fig. 2).

In vivo studies were then performed to further investigate the ability of lovastatin to increase pertuzumab binding to gastric tumors. Oral administration of lovastatin (8.3 mg/kg) to athymic mice bearing subcutaneous NCI-N87 gastric tumors was performed 12 h prior to and at the time of $^{89}$Zr-labeled pertuzumab injection [$^{89}$Zr]Zr-DFO-pertuzumab (4.44-5.18 MBq, 42-49 μg protein) [27]. The control cohort received saline orally instead of lovastatin. PET imaging studies were performed 4, 8, 24, and 48 h after injection of $^{89}$Zr-labeled pertuzumab. An increase in $^{89}$Zr-labeled pertuzumab uptake over time was observed in both groups (Fig. 2B,C). In the lovastatin-treated group, tumors could be
delineated at 4 h post-injection of pertuzumab. Biodistribution studies revealed that at 48 h post-injection of pertuzumab, tumor uptake of the radiolabeled analog of pertuzumab was higher in lovastatin-treated mice compared with mice in the control group (Fig. 2B, Supplementary Fig. 3 and 4). Tumors from control mice had an uptake of 18.4 ± 7.2 percent injected dose per gram (%ID/g) (n = 5), while tumors from lovastatin-treated mice yielded a tumor uptake of 32.1 ± 6.9 %ID/g (n = 5). Control experiments in HER2-positive/CAV1-positive NCI-N87 gastric xenografts demonstrated that the tumor uptake of a radiolabeled isotype control IgG was significantly low and comparable in both saline-treated mice as well as those treated with lovastatin [27]. Additional control studies in a HER2-negative/CAV1-positive MDA-MB-231 orthotopic mammary fat pad model demonstrated that 89Zr-labeled pertuzumab accumulation doesn’t increase upon lovastatin treatment in a HER2-negative tumor model (Supplementary Fig. 4 and 5).

Additional in vitro studies demonstrated that lovastatin-mediated increases in membrane-bound pertuzumab could be blocked with a 30-fold excess of pertuzumab in NCI-N87 cells (Fig. 3A). In addition, 89Zr-labeled pertuzumab was injected into athymic nude mice bearing subcutaneous NCI-N87 gastric tumors that were blocked with a 40-fold excess of unlabeled pertuzumab. ImmunoPET images and ex vivo biodistribution acquired at 48 h post-injection of antigen-blocked mice showed a significant reduction in tumor uptake of control mice (8.7 ± 0.4 %ID/g, n = 5) and lovastatin-treated mice (17.7 ± 3.5 %ID/g, n = 5) (Fig. 3B, C; Supplementary Fig. 3 and 4). Taken together, these studies support the potential of temporal lovastatin treatment to enhance the avidity of HER2-positive tumors for pertuzumab.
Trastuzumab-receptor blockade increases internalization of $^{89}$Zr-labeled pertuzumab

We also investigated the effects of unlabeled trastuzumab in modulating the tumor-targeting ability of radiolabeled pertuzumab in both control and lovastatin-treated gastric tumor cells and xenograft mice. Trastuzumab binds to the extracellular domain of HER2 at a different epitope than pertuzumab [41], and previous preclinical studies have demonstrated that pertuzumab affinity is enhanced in the presence of trastuzumab [24, 42]. We explored whether the increase in pertuzumab binding in the presence of trastuzumab was due to alterations in receptor endocytic trafficking. Cellular fractionation experiments were performed in control and lovastatin-treated cells incubated with $^{89}$Zr-pertuzumab in the presence and absence of a 30-fold excess of unlabeled trastuzumab. In lovastatin-treated cells, excess trastuzumab did not induce significant alterations in the amount of membrane or internalized radiolabeled pertuzumab (Fig. 4A). In comparison, the amount of $^{89}$Zr-labeled pertuzumab in the intracellular fraction was 1.7-fold higher in NCI-N87 control cells incubated with an excess of trastuzumab.

To further these findings, in vivo studies with unlabeled trastuzumab were conducted in both control and lovastatin-treated xenograft mice. The immunoPET images and ex vivo biodistribution in mice pretreated with lovastatin at 48 h post-injection of the radiotracer were very similar in the presence and absence of trastuzumab (Fig. 4B, Supplementary Fig. 3 and 4). For the control mice, tumor accumulation of $^{89}$Zr-labeled pertuzumab was significantly increased in the presence of trastuzumab ($47.5 \pm 9.3 \text{ %ID/g, } n = 5$). $^{89}$Zr is
described as a residualizing radiometal; the radiometal is trapped inside the cell after radioconstruct internalization and proteolytic degradation until it is externalized relatively slowly. Therefore, tumor uptake at 48 h post-injection is a result of not only receptor-bound but also internalized radioactivity. These results indicate that the increase in the amount of internalized $^{89}$Zr-labeled pertuzumab is a result of trastuzumab-mediated HER2 internalization, an effect that can be temporally modulated by treatment with lovastatin.

**Lovastatin allows $^{18}$F-based pretargeted PET imaging of HER2-positive tumors**

The rapid internalization of HER2 upon antibody binding is incompatible with pretargeted molecular imaging since it does not allow sufficient membrane-bound anti-HER2 antibody to be available for binding a radiolabeled small molecule. We hypothesized that our strategy of temporal modulation of the membrane bioavailability and stability of HER2 by treatment with lovastatin could extend the benefits of pretargeted immunoPET to this class of rapidly internalizing tumor-associated antigens (Fig. 5). Based on our data supporting lovastatin stabilization of HER2 at the cell surface, we predicted that antibody-mediated HER2 internalization would be lower in the presence of lovastatin compared to control mice, which will enhance pertuzumab binding to membranous HER2. As such, a radiolabeled small molecule (in our case $[^{18}$F]AIF-NOTA-PEG$_{11}$-Tz) would be able to conjugate bioorthogonally to trans-cyclooctene (TCO)-labeled antibody (trastuzumab or pertuzumab) bound to HER2. To explore this, we performed *in vivo* pretargeting experiments in nude mice bearing subcutaneous NCI-N87 gastric tumors. Based on our Western blot analysis showing that *in vivo* reduction of
CAV1 protein occurs between 12 and 48 h post-administration of lovastatin (Supplementary Fig. 7), our pretargeting experiments were conducted with a 24 h interval between the injection of trastuzumab-TCO or pertuzumab-TCO and radiolabeled tetrazine (Tz). In lovastatin-treated mice, $^{18}$F-PET images at 4 h post-injection of radiolabeled Tz clearly delineated HER2-positive tumors (Fig. 6; Supplementary Table 4). A pretargeting approach with trastuzumab-TCO and $[^{18}\text{F}]\text{AlF-NOTA-PEG}_{11}$-Tz (Supplementary Fig. 8) demonstrated increasing tumor uptake over time ($1.86 \pm 0.83 \%\text{ID/g at 30 min}, 3.08 \pm 0.50 \%\text{ID/g at 1.5 h, and } 4.20 \pm 1.03 \%\text{ID/g at 4 h}$) and decreasing blood radioactivity over time, from $6.01 \pm 0.41 \%\text{ID/g at 30 min to } 3.44 \pm 0.44 \%\text{ID/g at 4 h}$, in mice pretreated with lovastatin.

In pretargeting approaches using pertuzumab-TCO (Fig. 6), biodistribution data at 4 h post-injection of the radioligand revealed higher tumor uptake in lovastatin-treated mice ($[^{18}\text{F}]\text{AlF-NOTA-PEG}_{11}$- Tz, $3.30 \pm 0.04 \%\text{ID/g}$) compared to control mice ($[^{18}\text{F}]\text{AlF-NOTA-PEG}_{11}$-Tz, $1.20 \pm 0.02 \%\text{ID/g}$). Although tumor-to-blood ratios were higher after treatment with lovastatin, the results suggest that after 24 h not all pertuzumab-TCO had been cleared from the blood. Previous studies have demonstrated that an increase in the administration time between antibody-TCO and radiolabeled Tz improves the tumor-to-background contrast in pretargeting strategies [31]. In our study, such strategy was not feasible because CAV1 modulation with lovastatin is a transient effect, and CAV1 levels at 48 h post-administration of lovastatin are similar to those found in control tumors (Supplementary Fig. 7). These results suggest the potential of
pretargeted molecular imaging with anti-HER2 antibodies in HER2-positive tumors depleted of CAV1 protein.

**DISCUSSION**

Patient selection for therapy with anti-HER2 antibodies has been primarily based on HER2 positivity as assessed by total protein levels or gene amplification in tumor biopsy samples. This approach may not be reliable for gastric tumors, which display heterogeneous HER2 expression, and may contribute to the observed poor clinical response to pertuzumab/trastuzumab combinations in patients with gastric cancers [19]. Few studies have attempted to determine the extent to which receptor membrane dynamics affect HER2 detection and anti-HER2 antibody therapy. In previous work, we identified an inverse relationship between the expression levels of CAV1 and the presence of HER2 protein at the tumor cell membrane [27]. We showed that pharmacological modulation of CAV1 with lovastatin increases membrane HER2 availability for binding of trastuzumab in breast and gastric cancer cells. The use of lovastatin as a pharmacological modulator of CAV1 increased the uptake of trastuzumab even in tumors with non-predominant HER2 membrane staining.

In this study, we extended our prior work and gained further insights into how lovastatin affects HER2 stability at the cell membrane and binding of anti-HER2 antibodies to target cells. We found that lovastatin-induced accumulation of membrane HER2 is associated with an increase in the amount of non-activated HER2 dimers (Fig. 1). We
used molecular imaging to show that lovastatin increases membrane \textit{(in vitro)} and tumor \textit{(in vivo)} accumulation of pertuzumab (Figs. 2-4), a humanized HER2-targeted antibody that binds to the dimerization domain of HER2. Finally, using a pretargeted molecular imaging approach in NCI-N87 xenograft mice pretreated with lovastatin, we demonstrated that $^{18}$F-labeled Tz could delineate HER2-positive tumors despite the biological characteristics of HER2 as a circulating antigen and internalization of the TCO-conjugated antibody (Fig. 5, 6).

The cytoplasmic catalytic function of receptor tyrosine kinases is activated upon binding of a specific ligand to the monomeric receptor. This process induces receptor dimerization and autophosphorylation of tyrosine residues that in turn activates downstream signaling cascades [1, 5]. The PI3K-activated AKT pathway and p70S6K/p85S6K pathway are downstream signaling pathways activated by HER dimerization, and the Ras- and Shc-activated MAPK pathway is a target of all HER ligands [1]. Although there are no known ligands that bind to HER2, the receptor is activated upon dimerization with other members of the HER family [1, 5]. In gastric cancer, HER2 and HER3 overexpression and dimerization are associated with poor survival [43]. One mechanism that decreases downstream signaling mediated by the HER family involves ligand-mediated receptor endocytosis [1]. Preclinical studies have demonstrated that HER2 protein levels at the cell membrane are downregulated after treatment with trastuzumab due to an antibody-mediated internalization process [1]. CAV1 depletion leads to HER2 accumulation at the cell membrane and enhanced HER2-HER2 and HER2-EGFR dimer formation (Fig. 1). Furthermore, we found that the
increase in HER2-HER2 and HER2-EGFR dimers in response to lovastatin occurs without consequent receptor phosphorylation or activation of the MAPK pathway. Lovastatin increases HER2 dimerization without alteration in HER2 downstream oncogenic signaling plausibly due to the fact that our lovastatin pharmacological approach induced a transient effect in CAV1 depletion and HER2 availability at the cell membrane. Further studies are necessary to determine changes in CAV1 protein levels and HER2 membrane availability and downstream oncogenic signaling after treatment with single, fractionated and prolonged doses of lovastatin.

The presence of high levels of HER2 dimers on the cell surface could serve as an antibody trap to enhance binding with anti-HER2 antibodies. Pertuzumab is a HER2-targeted humanized antibody and it inhibits HER2-dimerization upon binding with domain II of the HER2 protein [40]. Pertuzumab is used clinically for the treatment of metastatic HER2-overexpressing breast cancer. Radiolabeled pertuzumab also enables preclinical [24] and clinical [22] non-invasive, antibody-directed imaging of HER2-positive breast cancer. Molecular imaging with $^{89}$Zr-labeled pertuzumab is not possible at early time points because of its accumulation in the liver and blood [22]. Indeed, PET imaging of BT474 breast cancer xenografts using $^{89}$Zr-labeled pertuzumab was only possible 120-168 h post administration of the radiotracer [24]. The clinical use of $^{89}$Zr-labeled pertuzumab required that breast cancer patients return to the clinic for a PET scan 5-8 days after radiotracer administration [22]. Our approach using lovastatin allowed tumor delineation at early time points and enhanced contrast 48 h after tracer injection; this strategy could improve the use of anti-HER2 antibodies for molecular imaging. The
ability of lovastatin in depleting CAV1 protein in a transient manner is also a potential pharmacological strategy in molecular imaging with antibody fragments and engineered variants as these biomolecules exhibit faster accumulation in the tumor tissue when compared with fully intact antibodies.

Trastuzumab is an anti-HER2 humanized antibody that, in combination with chemotherapy, is a first-line treatment for patients with gastric tumors [10]. However, trastuzumab has not been established as a second-line treatment of advanced gastric cancer [44]. In metastatic breast tumors, dual HER2 blockade using pertuzumab and trastuzumab is more effective in the inhibition of HER2 signaling than trastuzumab alone [45]. These agents are thought to have complementary activities – trastuzumab binds to domain IV of HER2 [41] and inhibits ligand-independent signaling [46], whereas pertuzumab binds to the HER2 dimerization domain II [40] to inhibit ligand-dependent signaling [47]. Preclinical studies have shown that the combination of trastuzumab and pertuzumab is more effective in HER2-positive human gastric cancer xenografts in comparison to either antibody alone [17]. However, in recent clinical trials, the combination of pertuzumab and trastuzumab did not improve outcomes in patients with metastatic or advanced gastric tumors [18]. This result may be due, in part, to limitations in the current strategies to select patients with HER2-positive gastric tumors. Patient selection for HER2-targeted therapies is currently based on tumor-specific amplification of the HER2 gene (positive fluorescence in-situ hybridization [FISH]) or overexpression of the HER2 protein (immunohistochemistry [IHC] score of 3+) [10]. Patient selection based on FISH and IHC has limitations when evaluating a heterogeneous target such as HER2. In comparison to breast tumors, IHC in gastric tumors shows that HER2 exhibits
heterogeneous [15] and incomplete membrane staining [48]. These studies support the need for improved strategies to identify patients with HER2-positive gastric tumors who might benefit from HER2-targeted therapies. Molecular imaging with $^{89}$Zr-labeled pertuzumab could, in the right context, improve patient selection for HER2-targeted therapies [22]. In addition, we have identified an association between low HER2 membrane staining and high CAV1 protein levels in gastric tumors [27], suggesting the potential role for CAV1 to be used as a complementary biomarker to improve the selection of gastric cancer patients who may benefit from treatment with anti-HER2 targeted therapies.

Molecular imaging studies have shown that tumor accumulation of $^{89}$Zr-labeled pertuzumab in breast tumors is enhanced in the presence of trastuzumab [24]. Our study found similar results in gastric tumors when $^{89}$Zr-labeled pertuzumab was administered in the presence of an excess of unlabeled trastuzumab. These observations can be explained by trastuzumab-induction of HER2 conformational changes that increase pertuzumab affinity for HER2 [42]. From the perspective of HER2 endocytosis, our findings suggest that trastuzumab not only induces conformational changes of the HER2 receptor, but it also enhances pertuzumab intracellular accumulation as a result of trastuzumab-mediated HER2 internalization.

PET imaging using $^{89}$Zr-labeled full-length antibodies results in unnecessarily high radiation exposure as compared to small molecules labeled with radionuclides with relatively short physical half-lives (e.g., $^{18}$F). Although pretargeted molecular imaging is
a potential alternative to $^{89}$Zr-labeled antibodies [33], this strategy is dependent on the presence of the target at the cell membrane. HER2 is not an ideal target for pretargeted PET approaches, given that it is a circulating antigen and is internalized through caveolae-mediated endocytic trafficking. Although there has been previous in vitro success of pretargeting of internalizing antibodies in HER2-positive cancer cells, trastuzumab internalization after binding to HER2 results in modest tumor uptake in an in vivo model [33]. In our studies, lovastatin increased tumor uptake of $^{18}$F-radiolabeled Tz in pretargeted imaging approaches using trastuzumab and pertuzumab (Fig. 5, 6; Supplementary Fig. 8). Further randomized preclinical investigation - using different doses of statin and combinations of statin/antibody - in HER2-positive tumors containing different levels of CAV1 protein and retrospective studies in patients receiving standard doses of statin for concurrent cardiovascular indications while being treated with anti-HER2 antibodies are necessary to determine if our pharmacologic strategy can outperform HER2-targeted imaging/therapy and pretargeted approaches.

In sum, our data on the role of endocytic trafficking in anti-HER2 antibody therapy support the need to consider HER2 membrane availability during patient selection for anti-HER2 therapies. Furthermore, our work indicates that HER2 membrane availability can be modulated with lovastatin to enhance binding of $^{89}$Zr-labeled pertuzumab in HER2-positive gastric cancer cells characterized by a non-predominant HER2 membrane staining. These findings are significant as they provide support for pharmacological modulation of CAV1 to improve pretargeted strategies for molecular imaging and therapy of HER2-positive gastric tumors. A limitation of our work is that we could not
increase tumor-to-background ratios by extending the injection time between pertuzumab and radiolabeled Tz for time points longer than 24 h. However, our studies provide a foundation for the use of a pharmacological approach to modulate HER2 localization and enhance pertuzumab tumor binding and pretargeted molecular imaging. Future preclinical studies combining pertuzumab plus lovastatin are planned to evaluate whether this combination can improve the therapeutic utility of anti-HER2 targeting in gastric tumors.
Financial disclosure: This research was funded in part through the NIH/NCI Cancer Center Support Grant P30 CA008748, NIH U01 CA221046, NIH R01 CA204167, the MSK Geoffrey Beene Cancer Research Center, a Tow Foundation Postdoctoral Fellowship from the MSK Center for Molecular Imaging and Nanotechnology (P.M.R.P.), and a Ruth L. Kirschstein National Research Service Award postdoctoral fellowship (L.M.C. NIH F32-EB025050).

Disclosure of potential conflicts of interest: In regards to this publication, Jason S. Lewis has received research reagents from Genentech and Y. Janjigian has received research funding from Genentech/Roche.

Acknowledgements
The authors gratefully acknowledge members of the MSKCC Small Animal Imaging Core Facility, the Radiochemistry and Molecular Imaging Probe Core. We gratefully acknowledge Mr. William H. and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research and The Center for Experimental Therapeutics at MSK. We thank Dr. Jan-Philip Meyer for sharing the tetrazine reagent used in the pretargeting experiments and Dr. Sai Kiran Sharma for critical review and comments on an early version of this manuscript.
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Figure 1. Lovastatin treatment increases HER2-HER2 and HER2-EGFR non-activated dimers in gastric cancer cells. (A, B) Western blot of EGFR, HER2, phosphorylated tyrosine (p-Tyr), phosphorylated MAPKs (p-MAPKs), and total MAPKs from total cell extracts or extracts obtained after immunoprecipitation (IP) with an anti-HER2 antibody. Untreated NCI-N87 gastric cancer cells served as a control. NCI-N87 cells were incubated with 25 μM of lovastatin for 4 h. β-actin was used as a loading control. Western blot quantification of EGFR and HER2 (normalized to control) are represented as mean ± SEM (*P < 0.05 based on a Student’s t test). The experiment was repeated three times.
Figure 2. Lovastatin treatment increases \textit{in vitro} membrane bound pertuzumab and \textit{in vivo} tumors’ avidity for pertuzumab. (A) Membrane-bound and internalized \([^{89}\text{Zr}]\text{Zr-DFO-pertuzumab}\) after treatment with lovastatin in NCI-N87 cancer cells. NCI-N87 untreated cells served as control. NCI-N87 cancer cells were incubated with 25 μM of lovastatin for 4 h before addition of 1 μM \(^{89}\text{Zr}\)-labeled pertuzumab for 1.5 h. Data represent the mean ± SEM (\(n=4\) experiments, \(*P<0.05\) based on a Student’s \(t\) test). (B) Biodistribution and (C) representative maximum intensity projection images (MIPs) and coronal PET images of \([^{89}\text{Zr}]\text{Zr-DFO-pertuzumab}\) in athymic nude mice bearing subcutaneous NCI-N87 gastric tumors treated with lovastatin. Lovastatin (8.3 mg/kg of mice) was orally administered 12 h prior and at the same time as the tail vein injection of
[\textsuperscript{89}Zr]Zr-DFO-pertuzumab (4.44-5.18 MBq, 42-49 μg protein). Control mice received oral saline instead of lovastatin. Biodistribution data represent the mean ± SEM (n = 5 mice per group, *P < 0.05 based on a Student’s t test).
Figure 3. Lovastatin-mediated increase in membrane-bound pertuzumab is blocked with an excess of pertuzumab. (A) Membrane-bound and internalized \[^{89}\text{Zr}]\text{Zr-DFO-pertuzumab\) in the presence of an excess of unlabeled pertuzumab after lovastatin treatment in NCI-N87 cells. Blocking experiments were performed by incubation with \[^{89}\text{Zr}]\text{Zr-labeled pertuzumab in the presence of a 30-fold excess of pertuzumab. Untreated NCI-N87 cells served as control. NCI-N87 cells were incubated with 25 μM of lovastatin for 4 h before addition of 1 μM \[^{89}\text{Zr}\)]\text{-labeled pertuzumab and incubation for 1.5 h. Data represent the mean ± SEM (}\, n = 4 \, \text{experiments, } ^* P < 0.05 \, \text{and } ^{**} P < 0.01 \, \text{based on a Student’s } t \text{ test). (B) Representative maximum intensity projection images (MIPs) and coronal PET images and (C) tumor uptake (at 48 h post-injection of \[^{89}\text{Zr}\)]\text{-labeled pertuzumab\) in athymic nude mice bearing subcutaneous NCI-N87 gastric tumors with and without blocking with unlabeled pertuzumab. Lovastatin (8.3 mg/kg) was orally administered 12 h prior and at the same time as the tail vein injection of \[^{89}\text{Zr}\)]\text{Zr-DFO-pertuzumab (4.44-5.18 MBq, 42-49 μg protein). Control mice received oral saline. Blocking experiments were performed by administration of \[^{89}\text{Zr}]\text{labeled pertuzumab in the presence of a 40-fold excess of pertuzumab. Data represent the mean ± SEM (}\, n = 5 \, \text{mice per group, } ^* P < 0.05 \, \text{and } ^{**} P < 0.01 \, \text{based on a Student’s } t \text{ test).}
**Figure 4. Trastuzumab-receptor blockade increases internalization of $^{89}$Zr-labeled pertuzumab.** (A) Membrane-bound and internalized $[^{89}\text{Zr}]$Zr-DFO-pertuzumab in the presence of trastuzumab in NCI-N87 cancer cells with or without lovastatin treatment. Blocking experiments were performed by incubation with $^{89}$Zr-labeled pertuzumab in the presence of a 30-fold excess of trastuzumab. NCI-N87 untreated cells served as control. NCI-N87 cancer cells were incubated with 25 μM of lovastatin for 4 h before addition of 1 μM $^{89}$Zr-labeled pertuzumab for 1.5 h. Data represent the mean ± SEM ($n = 4$ experiments, *$P < 0.05$ based on a Student’s $t$ test). (B) Representative coronal PET images and (C) tumor uptake (at 48 h post-injection of $^{89}$Zr-labeled pertuzumab) in athymic nude mice bearing subcutaneous NCI-N87 gastric tumors with and without blocking with unlabeled trastuzumab. Lovastatin (8.3 mg/kg of mice) was orally administered 12 h prior and at the same time as the tail vein injection of $[^{89}\text{Zr}]$Zr-DFO-pertuzumab (4.44-5.18 MBq, 42-49 μg protein). Control mice received oral saline. Blocking experiments were performed by administration of $^{89}$Zr-labeled pertuzumab in the presence of a 40-fold excess of trastuzumab. Data represent the mean ± SEM ($n = 5$ mice per group, ***$P < 0.001$ based on a Student’s $t$ test).
Figure 5. Schematic of a pretargeting approach to image gastric tumors with pertuzumab in the presence of lovastatin. Lovastatin depletes CAV1 increasing HER2 membrane availability and HER2 inactive dimers for binding pertuzumab. The TCO-labeled pertuzumab (slow pharmacokinetics) is injected days ahead of administrating a radiolabeled small molecule. Then, only hours before imaging, the administered radiolabeled small molecule travels through the blood rapidly, either clicking with the TCO-labeled antibody or quickly clearing from the patient. HER2 is represented in light blue and caveolin-1 in yellow.
Figure 6. Pretreatment of gastric tumor cells with lovastatin improves pretargeted molecular imaging. (A) Representative coronal PET images, (B) maximum intensity projection images (MIPs), and (C) biodistribution at 4 h post-injection of \([^{18}F]AIF\)-NOTA-PEG\(_{11}\)-Tz in athymic nude mice bearing subcutaneous gastric tumors. Lovastatin (8.3 mg/kg of mice) was orally administered 12 h prior and at the same time as the tail vein injection of pertuzumab-TCO. Mice were administered pertuzumab-TCO (0.42 nmol) 24 h prior to the injection of the \(^{18}F\)-labeled tracer (14.73-16.54 MBq, 0.83 nmol) via the tail vein. Data represent the mean ± SEM (\(n = 5\) experiments).
Supplementary Figure 1. Representative quality control (QC) radio-HPLC trace for $^{18}$F-labeled Tz, which was obtained with high purity (>98%) and high molar activity (55.5 GBq/µmol).

Supplementary Figure 2. Binding kinetics of $^{89}$Zr-labeled pertuzumab in control and lovastatin-treated NCI-N87 cells. NCI-N87 control and lovastatin-treated cells were incubated with $^{89}$Zr-labeled pertuzumab (0 to 256 nM) for 2 h at 4 °C (upper panel). Specific binding of $^{89}$Zr-labeled pertuzumab (red or black circles) and non-linear regression curve fit (dotted lines). Data are presented as mean ± S.E.M, n = 3. Binding parameters (lower panel) of $^{89}$Zr-labeled pertuzumab to NCI-N87 cells control or treated with lovastatin (*$P < 0.05$ based on a Student’s t-test).
Supplementary Figure 3. Biodistribution in control (CT) athymic nude mice bearing subcutaneous NCI-N87 gastric tumors with and without blocking with unlabeled pertuzumab or trastuzumab. \[^{89}\text{Zr}]\text{Zr-DFO-pertuzumab (4.44-5.18 MBq, 42-49 \(\mu\)g protein) was administered by tail vein injection. Blocking experiments were performed by administration of \[^{89}\text{Zr}\]labeled pertuzumab in the presence of a 40-fold molar excess of pertuzumab or trastuzumab. Biodistribution studies were performed at 48 h post-injection of \[^{89}\text{Zr}\]labeled pertuzumab. Data represent the mean ± SEM (\(n = 5\) mice per group, * \(P < 0.05\), ** \(P < 0.01\), and *** \(P < 0.01\) based on a Student’s \(t\) test). Tumor-to-organ ratios radioactivity are shown in Supplementary Table 2.
Supplementary Figure 4. Biodistribution in lovastatin-treated athymic nude mice bearing subcutaneous NCI-N87 gastric tumors with and without blocking with unlabeled trastuzumab or pertuzumab. Lovastatin (8.3 mg/kg) was orally administered 12 h prior to and at the same time as the tail vein injection of $[^{89}\text{Zr}]\text{Zr-DFO-pertuzumab}$ (4.44-5.18 MBq, 42-49 μg protein). Blocking experiments were performed by administration of $^{89}\text{Zr}$-labeled pertuzumab in the presence of a 40-fold molar excess of trastuzumab or pertuzumab. Biodistribution studies were performed at 48 h post-injection of $^{89}\text{Zr}$-labeled pertuzumab. Data represent the mean ± SEM, ($n$ = 5 mice per group, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ based on a Student’s $t$ test). Tumor-to-organ ratios radioactivity are shown in Supplementary Table 3.
Supplementary Figure 5. Representative coronal PET images of $^{89}$Zr-DFO-pertuzumab in athymic nude mice bearing orthotopic MDA-MB-231 mammary fat pad tumors treated with lovastatin. Lovastatin (8.3 mg/kg of mice) was orally administered 12 h prior and at the same time as the tail vein injection of $^{89}$Zr-DFO-pertuzumab (4.44-5.18 MBq, 42-49 μg protein). Control mice received oral saline instead of lovastatin.
**Supplementary Figure 6.** Biodistribution of $^{89}$Zr-labeled pertuzumab in lovastatin-treated athymic nude mice bearing orthotopic MDA-MB-231 mammary fat pad tumors. Lovastatin (8.3 mg/kg) was orally administered 12 h prior to and at the same time as the tail vein injection of $[^{89}\text{Zr}]\text{Zr-DFO-pertuzumab}$ (4.44-5.18 MBq, 42-49 μg protein). Biodistribution studies were performed at 48 h post-injection of $^{89}$Zr-labeled pertuzumab. Data represent the mean ± SEM, ($n = 3$ mice per group).
Supplementary Figure 7. Western blot of CAV1 in the total cell lysate of NCI-N87 subcutaneous tumors from athymic nude mice. Lovastatin (8.3 mg/kg) was orally administered twice, with an interval of 12 h between each administration. Tumor lysates were prepared at 0, 12, 16, and 48 h after lovastatin administration and analyzed by Western blot.

| Time (h) | 0 | 12 | 16 | 48 |
|----------|---|----|----|----|
|          | ![CAV1](image1) | ![CAV1](image2) | ![CAV1](image3) | ![CAV1](image4) |
|          | ![β-actin](image5) | ![β-actin](image6) | ![β-actin](image7) | ![β-actin](image8) |
Supplementary Figure 8. (A, C) Representative maximum intensity projection PET images (MIPs) and (B, D) biodistribution data at 0.5, 1.5, and 4 h post-injection of $^{18}$FAlF-NOTA-PEG<sub>11</sub>-Tz in athymic nude mice bearing subcutaneous gastric tumors. Lovastatin (8.3 mg/kg) was orally administered 12 h prior to and at the same time as the tail vein injection of trastuzumab-TCO, and then at the same time as the tail vein injection of $^{18}$FAlF-NOTA-PEG<sub>11</sub>-Tz. Mice were given trastuzumab-TCO (0.42 nmol) 24 h prior to injection of the $^{18}$F-labeled tracer (10.36-11.1 MBq, 0.83 nmol) via the tail vein. Control (CT) mice received with PBS instead of lovastatin. Biodistribution data represent the mean ± SEM for five mice.
**Supplementary Table 1.** NCIN87 tumor-to-organ ratios at 48 h after injection of $^{89}$Zr-pertuzumab in control and lovastatin-administrated mice.

|                | Control | Lovastatin |
|----------------|---------|------------|
| Blood          | 1.36    | 2.27       |
| Heart          | 3.64    | 5.98       |
| Lung           | 2.45    | 3.85       |
| Liver          | 2.94    | 8.05       |
| Spleen         | 5.16    | 6.17       |
| Pancreas       | 13.75   | 15.84      |
| Stomach        | 10.81   | 23.25      |
| Kidneys        | 3.46    | 5.77       |
| Small intestine| 12.41   | 20.48      |
| Large intestine| 25.39   | 41.55      |
| Bone           | 9.49    | 7.92       |
| Muscle         | 16.37   | 20.48      |
| Tail           | 7.50    | 10.55      |
| Tumor          | 1.00    | 1.00       |
| Skin           | 5.01    | 7.17       |

**Supplementary Table 2.** NCIN87 tumor-to-organ ratios at 48 h after injection of $^{89}$Zr-pertuzumab in control mice after blocking with unlabeled trastuzumab or pertuzumab.

|                | Control | Control + Blocking Trastuzumab | Control + Blocking Pertuzumab |
|----------------|---------|-------------------------------|------------------------------|
| Blood          | 1.36    | 7.39                          | 0.73                         |
| Heart          | 3.64    | 22.39                         | 1.55                         |
| Lungs          | 2.45    | 15.55                         | 2.14                         |
| Liver          | 2.94    | 35.41                         | 2.45                         |
| Spleen         | 5.16    | 36.05                         | 2.11                         |
| Pancreas       | 13.75   | 90.26                         | 8.06                         |
| Stomach        | 10.81   | 168.69                        | 11.29                        |
| Small Intestine| 3.46    | 91.56                         | 9.05                         |
| Large Intestine| 12.41   | 132.13                        | 12.40                        |
| Kidneys        | 25.39   | 22.87                         | 2.71                         |
| Muscle         | 9.49    | 155.00                        | 6.28                         |
| Bone           | 16.37   | 31.23                         | 2.36                         |
| Tumor          | 1.00    | 1.00                          | 1.00                         |
| Skin           | 7.50    | 26.96                         | 3.61                         |
| Tail           | 5.01    | 22.50                         | 1.63                         |
**Supplementary Table 3.** NCIN87 tumor-to-organ ratios at 48 h post-injection of $^{89}$Zr-pertuzumab in statin-treated mice after blocking with unlabeled trastuzumab or pertuzumab.

|                     | Statin + Blocking Trastuzumab | Statin + Blocking Pertuzumab |
|---------------------|------------------------------|------------------------------|
| Blood               | 2.27                         | 2.39                         |
| Heart               | 5.98                         | 7.99                         |
| Lung                | 3.85                         | 4.17                         |
| Liver               | 8.05                         | 9.04                         |
| Spleen              | 6.17                         | 22.21                        |
| Pancreas            | 15.84                        | 24.40                        |
| Stomach             | 23.25                        | 28.72                        |
| Kidneys             | 5.77                         | 7.39                         |
| Small Intestine     | 20.48                        | 34.20                        |
| Large Intestine     | 41.55                        | 35.51                        |
| Bone                | 7.92                         | 18.31                        |
| Muscle              | 20.48                        | 50.29                        |
| Tail                | 10.55                        | 11.74                        |
| Tumor               | 1.00                         | 1.00                         |
| Skin                | 7.17                         | 20.91                        |

**Supplementary Table 4.** NCIN87 tumor-to-organ ratios at 4 h post-injection of $[^{18}$F$]AIF-NOTA-PEG_{11}$-Tz in control and statin-treated mice administrated with pertuzumab-TCO.

|                   | Control | Lovastatin |
|-------------------|---------|------------|
| Blood             | 0.13    | 0.48       |
| Heart             | 0.41    | 1.32       |
| Lung              | 0.31    | 0.97       |
| Liver             | 0.57    | 1.74       |
| Spleen            | 0.82    | 2.73       |
| Pancreas          | 1.08    | 3.81       |
| Stomach           | 1.33    | 4.14       |
| Kidneys           | 0.28    | 0.88       |
| Small Intestine   | 0.53    | 2.57       |
| Large Intestine   | 4.68    | 4.97       |
| Bone              | 0.70    | 2.71       |
| Muscle            | 2.51    | 4.75       |
| Tail              | 0.72    | 2.09       |
| Tumor             | 1.00    | 1.00       |
| Skin              | 1.23    | 3.87       |
Temporal Modulation of HER2 Membrane Availability Increases Pertuzumab Uptake and Pretargeted Molecular Imaging of Gastric Tumors

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J Nucl Med.
Published online: June 6, 2019.
Doi: 10.2967/jnumed.119.225813

This article and updated information are available at:
http://jnm.snmjournals.org/content/early/2019/06/06/jnumed.119.225813

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