SUPPLEMENTAL DATA

Cell Culture and Cell Treatments

Human gastric cancer cell line NCIN87 was purchased from American Type Culture Collection (ATCC). NCIN87 cancer cells were grown in Roswell Park Memorial Institute (RPMI)-1640 growth medium supplemented with 10% fetal serum bovine (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin. The epidermoid carcinoma cells A431 were obtained from Dr. Jason Lewis at Memorial Sloan Kettering Cancer and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 4.5 g/L glucose, 100 U/mL penicillin, and 100 µg/mL streptomycin.

For all *in vitro* and *in vivo* experiments, NCIN87 or A431 cancer cells were used within passage 5-12. Cell lines were authenticated using short tandem repeat analysis.

Cell were incubated with a working solution of metformin of 5 mM prepared in the respective complete cell culture media. The working solution of 5 mM metformin was prepared from a 50 mM stock solution of metformin (Sigma-Aldrich).

Western blot analysis

Whole-protein extracts were prepared from NCIN87 or A431 cancer cells after cell scraping in radioimmunoprecipitation assay (RIPA) buffer (Sodium chloride [150 mM], Tris hydrochloride [50 mM, pH 7.5], Ethylene glycol tetraacetic acid [5 mM], Triton X-100 [1%], Sodium deoxycholate [0.5%], Sodium dodecyl sulfate [0.1%], Phenylmethanesulfonyl [2 mM], Iodoacetamide [2 mM], Protease inhibitor cocktail [1x]). Following protein extraction, samples were denatured in Laemmli buffer (Invitrogen). Following protein electrophoresis (NuPage 4-12%

Bis-Tris protein gels, Invitrogen) and transfer to polyvinylidene difluoride (PVDF) membranes (Bio-Rad), the membranes were incubated in 5% w/v bovine serum albumin (BSA) or 5% non-fat dry milk in Tris-Buffered Saline buffer-Tween (TBS-T, Cell Signaling Technology) and probed with mouse anti β-actin 1:10,000 (Sigma, A1978), rabbit anti-HER2 1:800 (Abcam, ab131490), rabbit anti-HER2 phospho Y1139 1:500 (Abcam, ab53290), rabbit anti-CAV1 1:500 (Abcam, ab2910), rabbit anti-EGFR 1:1000 (Abcam, ab52894), or rabbit anti-EGFR phospho Y1068 1:500 (Abcam, ab40815). After washing the membranes with TBS-T, the membranes were incubated with Goat anti-Rabbit IgG(H+L) AlexaFluor™ Plus 680 (Invitrogen) or Goat anti-Mouse IgG(H+L) AlexaFluor™ Plus 800 (Invitrogen) and imaged on the Odyssey Infrared Imaging System (LI-COR Biosciences). Densiometric analysis of the respective bands was performed using ImageJ/FIJI (NIH, USA; https://imagej.net/Fiji). Uncropped scans of the blots are shown in Supplemental Fig. 1,3,4.

Biotinylation

Cell-surface proteins: For biotin pull-down assays, cells were incubated with 0.5 mg/mL of EZ-LINK Sulfo-NHS-Biotin (Thermo Fisher Scientific) for 30 min at 4 °C with gentle rotation and protected from light. The reaction was stopped by washing twice with 100 mM glycine (Thermo Fisher Scientific) in PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$. Protein lysates were prepared in RIPA as described in the section above. To collect the biotinylated proteins, an equal amount of biotinylated proteins were incubated with NeutrAvidin Agarose Resins (Thermo Fisher Scientific) overnight at 4 °C with gentle rotation. Samples were washed with RIPA buffer before suspension in Laemmli buffer and western blot analyses.

Endocytosis of biotinylated cell-surface proteins: After cell surface-biotinylation, the endocytosis of membrane proteins was promoted by the addition of 1 μM trastuzumab or 1 μM panitumumab in complete media at 37 °C during 90 min. The non-internalized cell-surface biotinylated proteins were removed by incubating cells with 50 mM Tris-HCl pH 8.7 [containing 20 mM dithiothreitol (DTT), 100 mM NaCl, 2.5 mM $CaCl_2$] for 20 min at 4 °C. After collecting protein lysates in RIPA buffer, the biotinylated internalized proteins were collected in NeutrAvidin agarose resins and Western blot analyses was performed as described above.

Conjugation of trastuzumab or panitumumab with Alexa-Fluor

Conjugation of trastuzumab or panitumumab with the fluorescent dyes Alexa Fluor 594 (Thermo Fisher Scientific, A20004) or Alexa Fluor 488 (Thermo Fisher Scientific, A20000) was performed at a molar ratio of 1:3 in an amine-free solution (1X PBS, pH 8.6) at 37° C. After reaction for 1.5 h, the conjugates were purified with a size exclusion column (PD-10; GE Healthcare). The immunoconjugates were further concentrated using a 50,000 g/mol molecular weight cut-off Amicon-filter. The protein concentration was calculated by measuring the absorption with the UV–Vis system at 280 nm or using the Pierce BCA assay (Thermo Fisher Scientific, 23227).

Conjugation and radiolabeling of IgG, trastuzumab or panitumumab

^{89}Zr-labeled antibody: ^{89}Zr-DFO-trastuzumab, ^{89}Zr-DFO-panitumumab, or control ^{89}Zr-DFO-IgG were obtained by first conjugating the antibodies with the chelate *p*-isothiocyanatobenzyl-desferrioxamine (DFO-Bz-NCS; Macrocyclics, Inc). ^{89}Zr-oxalate was obtained from the WUSTL Isotope Production Team and used to prepare the ^{89}Zr-labeled

trastuzumab or panitumumab following previously reported methods (*1-3*). Radiochemical purity (RCP) was determined by instant thin-layer chromatography (iTLC). The radiolabeled immunoconjugates used for *in vitro* and *in vivo* studies had a radiochemical purity of 99%, radiochemical yields ranging from 97-99%, specific activities of 22 MBq/nmol, and immunoreactivities above 95% (Supplemental Fig. 6,7).

^{64}Cu-labeled antibody: Copper-64 was obtained from WUSTL Isotope Production Team. Trastuzumab antibody was buffer exchanged and concentrated in 0.1 M of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.5) using the PD10 size exclusion column. Later, the trastuzumab antibody was reacted with a 20-fold molar excess of 2-S-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*-SCN-Bn-NOTA, Macrocyclics) for overnight at 4 °C with gentle agitation. The trastuzumab-NOTA conjugates were purified and concentrated in 0.1 M ammonium acetate buffer (pH 6) and radiolabeled with copper-64 supplied in ammonium acetate for 1 h at 37 °C, 450 rpm. The radiochemical yield and radiochemical purity was 100% and 98-99%, respectively, as determined by iTLC using the mixture of 0.1 M ammonium acetate buffer (pH 6) with 50 mM ethylenediaminetetraacetic acid as the mobile phase.

Internalization assays

Cells were pre-incubated with or without metformin for the indicated periods of time. Cells were then washed and incubated with PBS containing 1% w/v human serum albumin (HSA, Sigma Aldrich) and 0.1% w/v sodium azide (NaN_3, Sigma Aldrich) in the presence of 2.5 μg of ^{89}Zr-DFO-Antibody for 90 min at 37 °C. PBS containing unbound radioimmunoconjugate was removed, and the cells were washed twice with cold PBS. Cell surface-bound radiotracer was

collected by incubating cells at 4 °C for 15 min in a 50 mM glycine buffer containing 150 mM NaCl at pH 2.8. Internalized antibody was obtained after cell lysis with 1 M sodium hydroxide (NaOH). Finally, the three fractions were measured for radioactivity on a gamma counter calibrated for ^{89}Zr.

Immunofluorescence microscopy and ELISA experiments

Cells grown on coverslips were treated with 5 mM metformin for 2 h. Cells were then incubated with 1 μM of fluorescently labeled trastuzumab or panitumumab for 120 min at 37 °C in the presence or absence of metformin. Cells were then fixed with 4% paraformaldehyde (pH 7.4) before incubation with DAPI (1:2,000) for 10 min. The cells were imaged using a 60X oil immersion objective on the EVOS M5000 Imaging System (Invitrogen).

ELISA experiments were performed in NCIN87 cancer cells treated with 5 mM of metformin for 2 h using a sandwich kit and following the manufacturer's instructions (HER2 human ELISA kit, ThermoFisher Scientific).

Tumor xenografts

All animal experiments were performed following the guidelines approved by the Research Animal Resource Center and Institutional Animal Care and Use Committee at Washington University School of Medicine. HER2-expressing NCIN87 cells (5 million) or EGFR-expressing A431 cells (5 million) were injected subcutaneously (50 μL cell suspension / 50 μL of reconstituted basement membrane Matrigel) in the right shoulder of eight- to 10-week-old *nu/nu* female mice (Charles River Laboratories). NCIN87 tumors were allowed to grow until they reached approximately 100 mm^3 or 250-300 mm^3 for A431 tumors, as measured by external vernier caliper

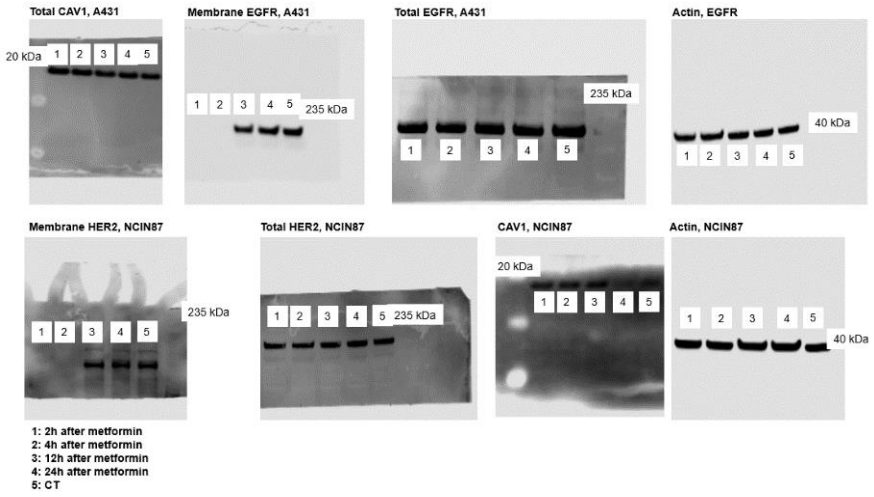
and applying the equation $V = (4\pi/3)$ x $(\alpha/2)^2$ x $(b/2)$. V is tumor volume (mm^3), α is the longest axis (mm), and b is the axis perpendicular to the longest axis (mm).

Mice were randomized into the following groups: control, acute dose of metformin, and a daily dose of metformin. Acute and daily administrations of metformin were performed as described in the next paragraphs. Researchers were blinded for which animal received saline, acute, or daily metformin administrations. ^{89}Zr-DFO-trastuzumab or ^{89}Zr-DFO-panitumumab (6.66-11.84 MBq, 80 μg of antibody), or ^{64}Cu-NOTA-trastuzumab (6.66-8.3 MBq or 45-56 μg protein) were administered via tail vein injection.

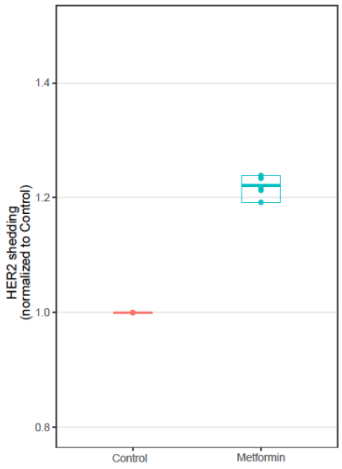
Acute metformin administration: Metformin (250 mg/kg) was orally administered 12 h prior to and at the same time as the tail vein injection of ^{89}Zr-DFO-antibody. A dose of 250 mg/kg of metformin corresponds to a 1,219 mg human dose.

Daily metformin administration: Daily administration of metformin was performed in NCIN87 or A431 xenografts. Metformin (200 mg/kg) was intraperitoneally administered for 7-11 consecutive days prior to the tail vein injection of ^{89}Zr-DFO-trastuzumab or ^{89}Zr-DFO-panitumumab. A dose of 200 mg/kg of metformin corresponds to a 975.2 mg human dose, and previous studies have shown this dose to be non-toxic in preclinical models (*4*).

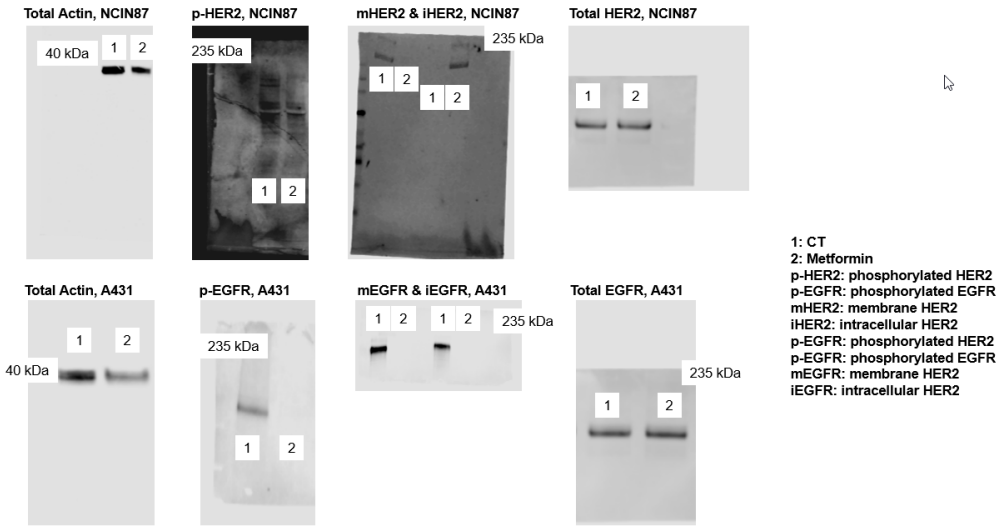
Metformin rebound study: Rebound of HER2 receptor in the presence and absence of metformin was studied in NCIN87 xenografts. The pre-metformin PET images were acquired using the short-lived ^{64}Cu-NOTA-trastuzumab at 24 h post tail vein injection. For on-metformin PET images, metformin (200 mg/kg) was intraperitoneally administered for 7 consecutive days prior to the tail vein injection of ^{64}Cu-NOTA-trastuzumab. Later, the metformin was ceased in the mice under therapy for at least 7 days prior to PET.



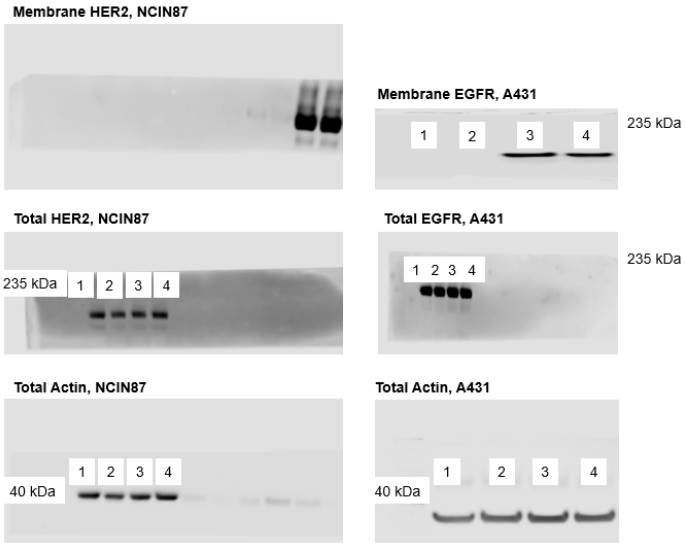
Supplemental Figure 1. Raw data of Western blot images shown in Figure 1A,B.



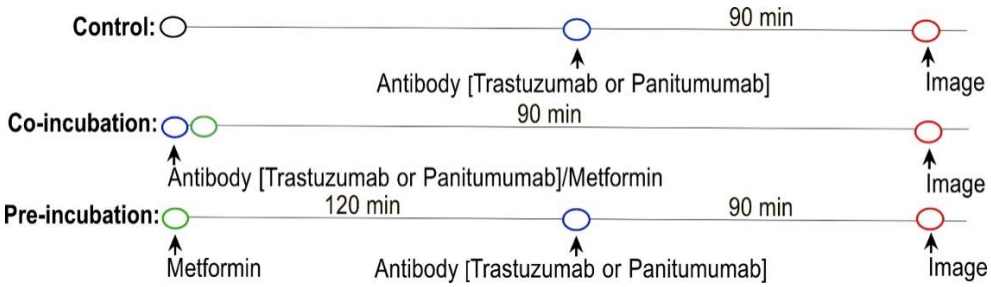
Supplemental Figure 2. Incubation of HER2$^+$ NCIN87 cancer cells with metformin for 2 h does not alter HER2 shedding as determined by ELISA experiments (Bars, n=4, mean ± SD).



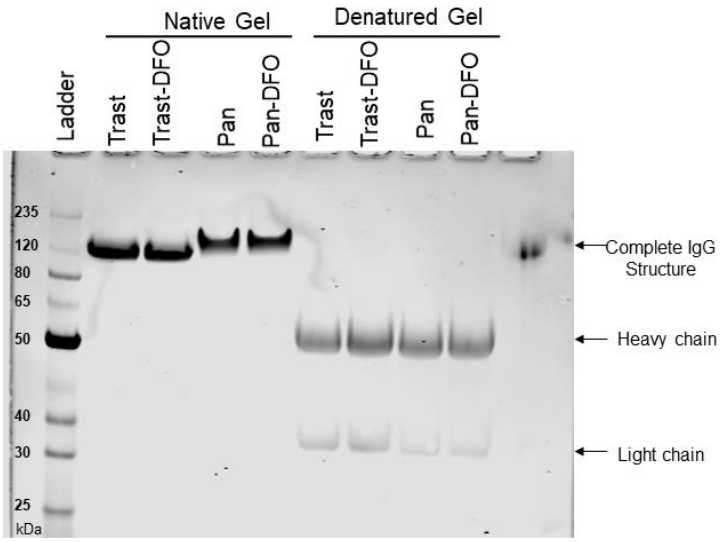
Supplemental Figure 3. Raw data of Western blot images shown in Figure 2B,C.



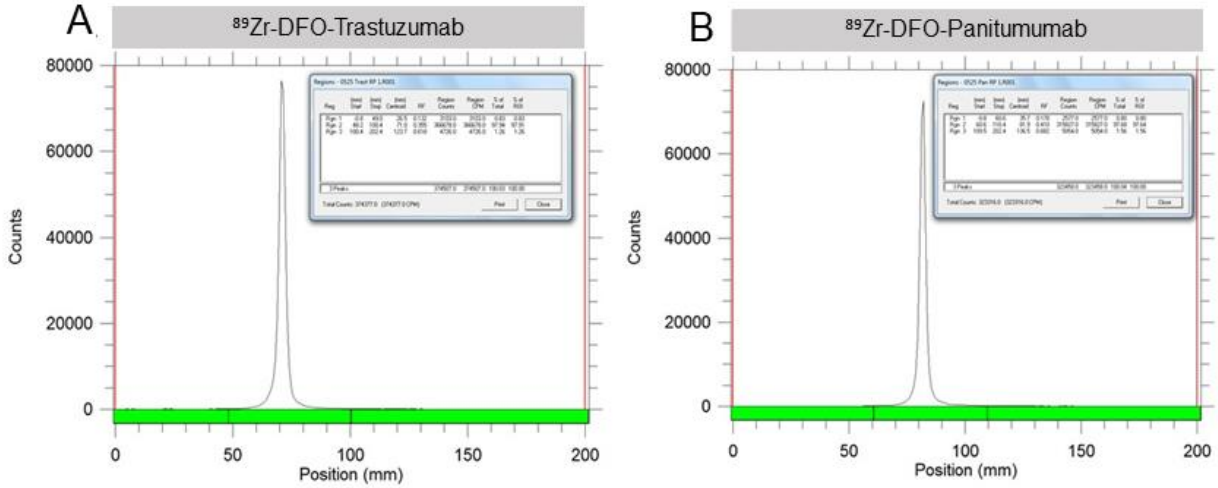
Supplemental Figure 4. Raw data of Western blot images shown in Figure 2B,C.



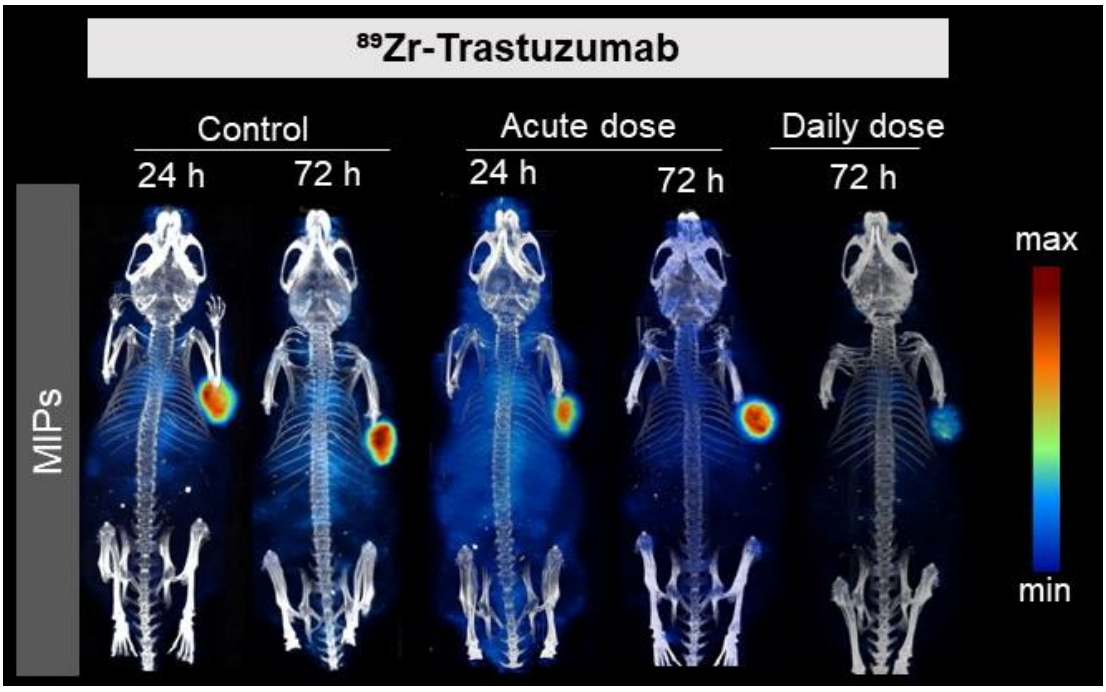
Supplemental Figure 5. Schematic representation of $HER2^{+}$ NCIN87 and $EGFR^{+}$ A431 cancer cells pre-treatment or co-treatment with metformin before incubation with fluorescently labeled trastuzumab or panitumumab. In pre-incubation schedules, NCIN87 or A431 cancer cells were incubated with 5 mM metformin for 2 h, washed with fresh culture media, and then incubated with 1 μM of Alexafluor conjugated trastuzumab or panitumumab for 90 min. In the co-incubation experiments, cancer cells were incubated with 1 μM of fluorescent antibodies for 90 min in the presence of 5 mM metformin.



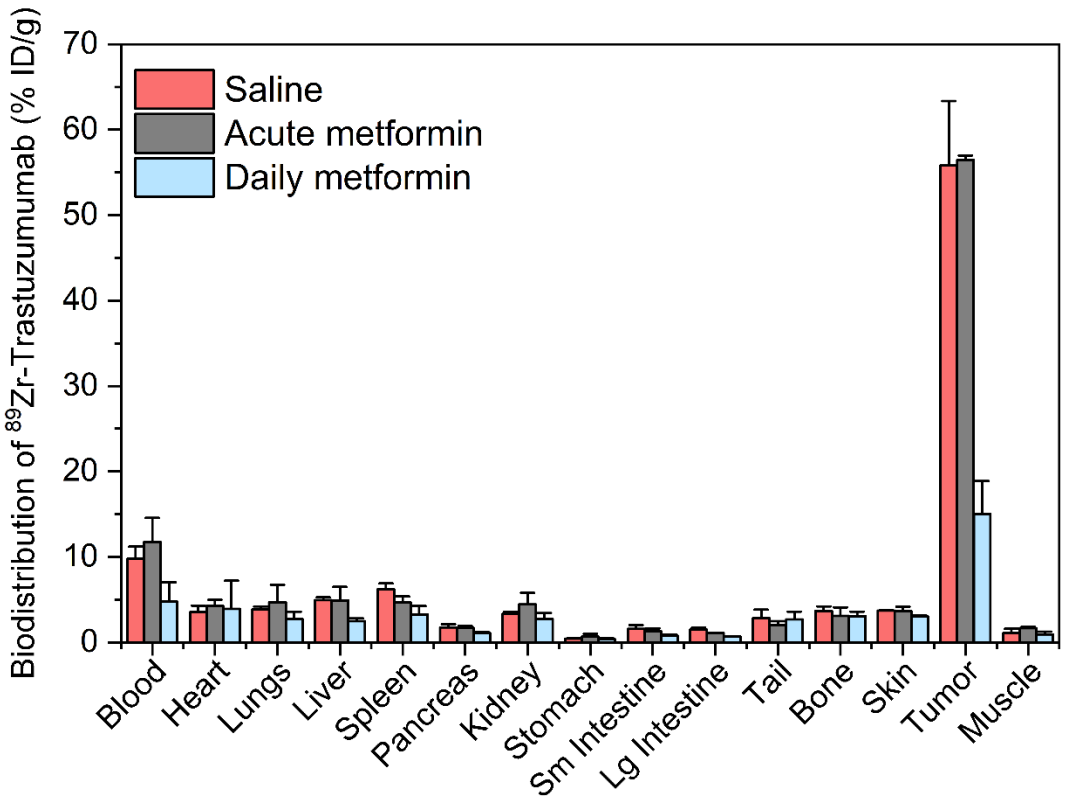
Supplemental Figure 6. Coomassie blue staining of SDS-PAGE gel (4-12%) of trastuzumab (Trast), trastuzumab-DFO (Trast-DFO), panitumumab (Pan), and panitumumab -DFO (Pan-DFO) performed under native and denatured conditions.



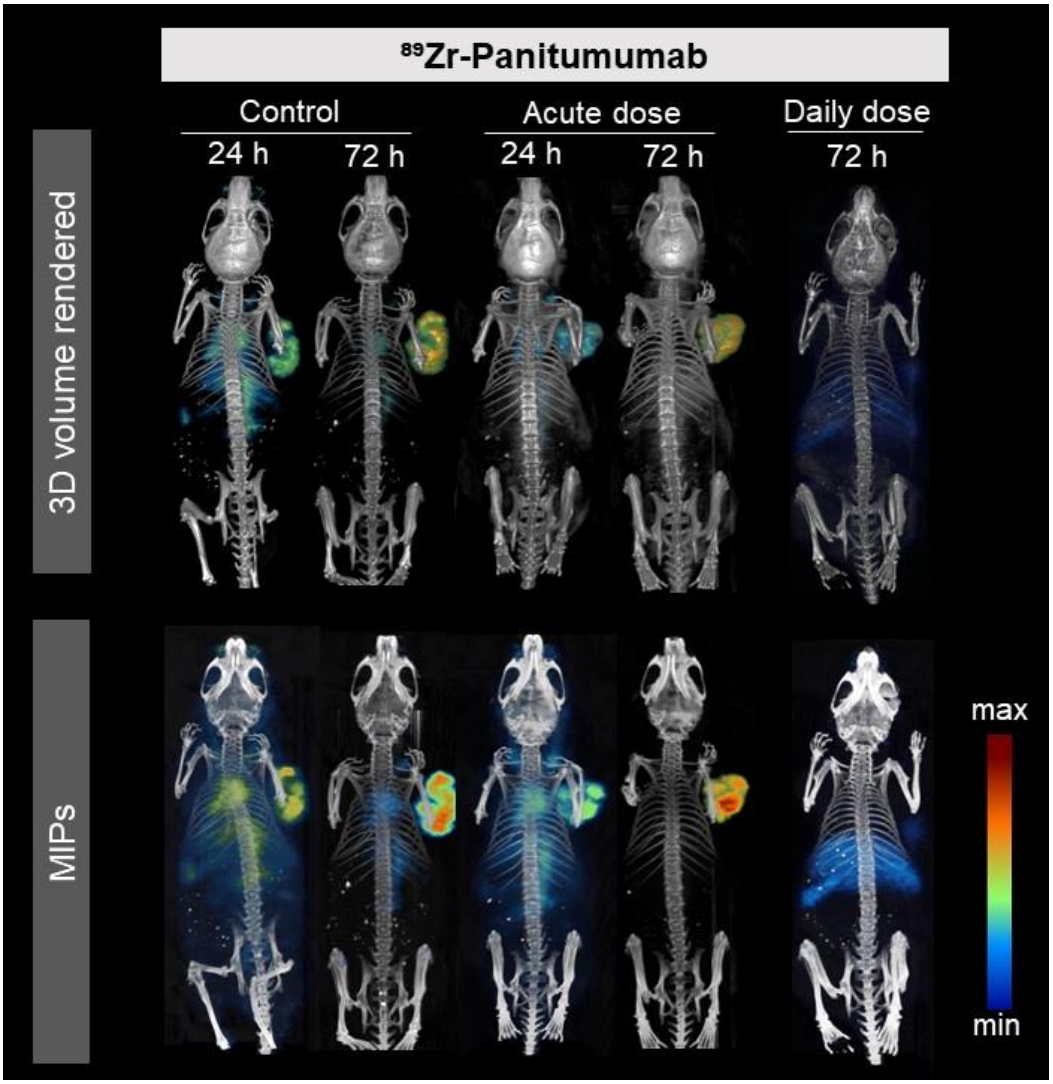
Supplemental Figure 7. Radiochemical purity of ^{89}Zr-DFO-trastuzumab and ^{89}Zr-DFO-panitumumab by instant thin layer chromatography (iTLC) of the radiotracer developed for the study. The inset shows the radiochemical yields of both tracers ~97%.



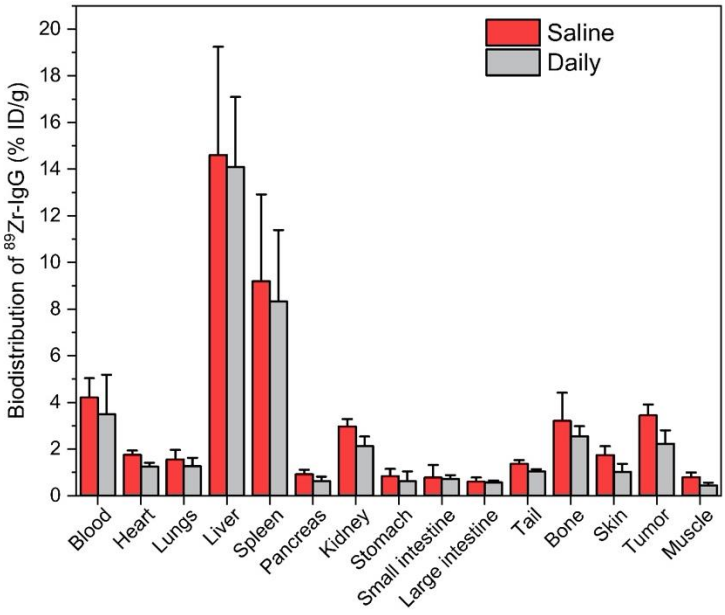
Supplemental Figure 8. Maximum Intensity Projection (MIPs) images of PET at 24 and 72 h post tail vein injection of ^{89}Zr-labeled trastuzumab. PET-CT images were acquired at 24 and 72 h post-injection of ^{89}Zr-DFO-trastuzumab in mice bearing HER2^{+} NCIN87 xenografts. Metformin (250 mg/kg) was orally administrated one day prior and at the same time as ^{89}Zr-DFO-trastuzumab (6.66-7.40 MBq, 45-80 μg protein) in the acute dose cohort. In the daily dose cohort, metformin (200 mg/kg) was injected intraperitoneally.



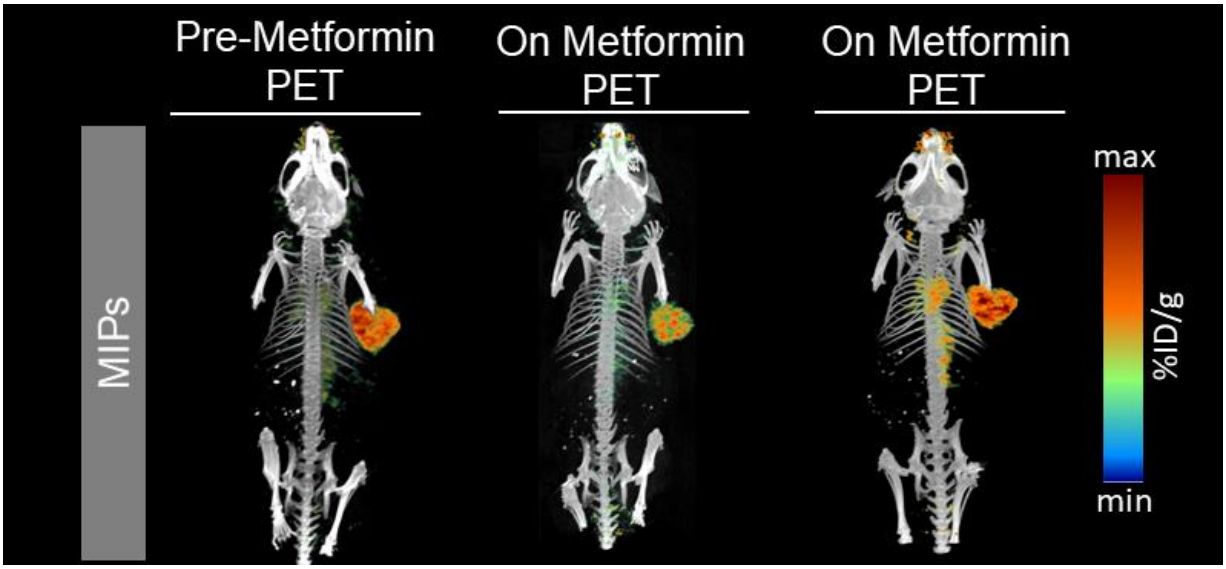
Supplemental Figure 9. Biodistribution data in control, acute dose (two doses of metformin by oral gavage), and daily dose (11 intraperitoneal injections of metformin; 200 mg/kg) cohorts of mice bearing s.c. $HER2^+$ NCIN87 tumors at 72 h p.i. of ^{89}Zr-DFO-trastuzumab. Acute dose cohort: metformin (250 mg/kg) was orally administrated one day prior and at the same time as the tail vein injection of ^{89}Zr-DFO-trastuzumab (6.66-7.40 MBq, 45-80 μg protein). In the daily dose cohort, metformin (200 mg/kg) was injected intraperitoneally. Bars, n = 3, mean±SD.



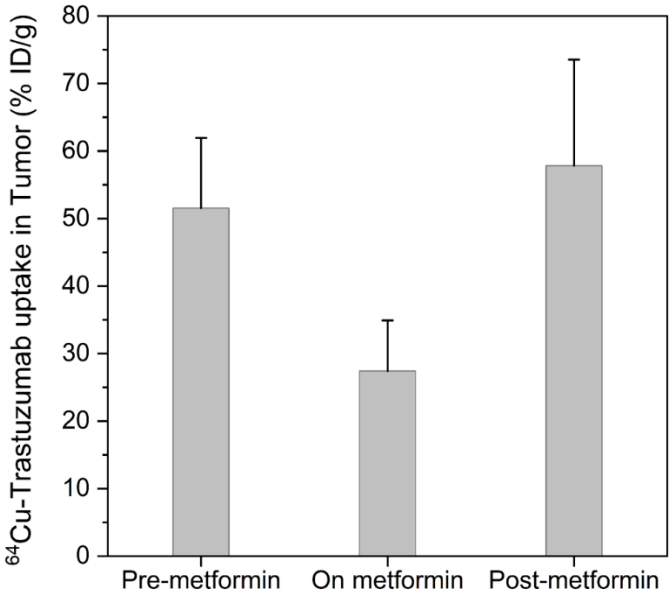
Supplemental Figure 10. 3D volume rendered and Maximum Intensity Projection (MIPs) images of PET at 24 and 72 h post tail vein injection of ^{89}Zr-labeled panitumumab. PET-CT images were acquired at 24 and 72 h post-injection of ^{89}Zr-DFO-panitumumab in mice bearing EGFR^{+} A431 xenografts. Metformin (250 mg/kg) was orally administrated one day prior and at the same time as ^{89}Zr-DFO-panitumumab (6.66-7.40 MBq, 45-80 µg protein) in the acute dose cohort. In the daily dose cohort, metformin (200 mg/kg) was injected intraperitoneally.



Supplemental Figure 11. Biodistribution data of ^{89}Zr-DFO-IgG in control and daily dose (seven intraperitoneal injections of metformin; 200 mg/kg) cohorts. In the daily dose cohort, the metformin (200 mg/kg) was injected intraperitoneally for five consecutive days, at the same time as the control ^{89}Zr-DFO-IgG (6.66-7.40 MBq, 80 μg protein) followed by two more doses of metformin prior and on the second imaging study. Bars, $n = 3$ mice per group, mean ± SD. %ID/g, percentage of injected dose per gram of tissue.



Supplemental Figure 12. Maximum Intensity Projection (MIPs) images of PET for the HER2 rebound study. For pre-metformin PET cohorts, the mice bearing $HER2^{+}$ NCIN87 were imaged at 24 h post tail vein injection of ^{64}Cu-labeled trastuzumab. For on metformin cohorts, metformin (200 mg/kg) was injected intraperitoneally for seven consecutive days: five injections were performed before administering the radiolabeled antibody, one injection at the same time and another at one day after administering ^{64}Cu-trastuzumab. On-metformin PET, the metformin treatment was ceased for one week, and the mice were imaged at 24 h after after tail vein injection of ^{64}Cu-labeled trastuzumab.



Supplemental Figure 13. ^{64}Cu-trastuzumab uptake in the mice before metformin treatment (pre-metformin), during treatment (on metformin), and after treatment (post-metformin). The ^{64}Cu-trastuzumab uptake was calculated based on the region of interest (ROI) drawing based on the PET using 3D Slicer software (version 5.0.3, a free and open source software https://www.slicer.org/). Bars, n = 3, mean±SD.

References

1. Pereira PMR, Sharma SK, Carter LM, et al. Caveolin-1 mediates cellular distribution of HER2 and affects trastuzumab binding and therapeutic efficacy. *Nat Commun.* 2018;9:5137.

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3. Pereira PMR, Mandleywala K, Ragupathi A, Lewis JS. Acute statin treatment improves antibody accumulation in EGFR- and PSMA-expressing tumors. *Clin Cancer Res.* 2020;26:6215-6229.

4. Cha JH, Yang WH, Xia W, et al. Metformin promotes antitumor immunity via endoplasmic-reticulum-associated degradation of PD-L1. *Mol Cell.* 2018;71:606-620.