MATERIALS AND METHODS

Conjugation and labeling of AMG 110 and Mec14.

AMG 110 and Mec14 were first purified with water for injections using a Vivaspin-2 10 kDa filter (GE Healthcare). Conjugation was performed by allowing BiTE® antibody constructs to react with a 6.7-fold molar excess of *N*-succinyldesferrioxamine-B-tetrafluorphenol (N-sucDf-TFP, ABX), as described earlier *(1)*. Conjugated BiTE® was radiolabeled on the same day with ^{89}Zr-oxalate (PerkinElmer). Free ^{89}Zr was removed by using a Vivaspin-2 10 kDa filter.

For labeling with respectively IRDye 800CW and 680RD, purified AMG 110 and Mec14 reacted with a 3-fold molar excess of IRDye as described earlier *(2)*. Unreacted dye was removed using PD10 desalting columns (Fisher Scientific).

Quality control of ^{89}Zr-AMG 110 and ^{89}Zr-Mec14.

Aggregation, fragmentation and concentration of ^{89}Zr-AMG 110, 800CW-AMG 110, ^{89}Zr-Mec14 and 680RD-Mec14 were assessed using size exclusion high performance liquid chromatography (SE-HPLC). The Waters SE-HPLC system was equipped with a dual wavelength absorbance detector, an in line radioactivity detector and a size exclusion column (Superdex 75 10/300 GL column; GE Healthcare). PBS was used as mobile phase at a flow of 0.7 ml/min. Radiochemical purity (RCP) of ^{89}Zr-AMG 110 and ^{89}Zr-Mec14 was determined using trichloroacetic acid precipitation *(3)*.

The *in vitro* binding characteristics (immunoreactive fraction; IRF) of the radiolabeled BiTE® antibody constructs were determined in a cell binding assay as described by Lindmo et al. *(4)*. In short, two series of HT-29 cell dilutions were incubated in duplicate with 15 ng/mL ^{89}Zr-AMG 110 for 2 h at 4°C while shaking. To

one of the cell series a 1000 fold excess of cold AMG 110 was added to block EpCAM specific binding and set the reference used to correct for non-specific binding. After 2 h incubation, cells were washed twice with PBS containing 1% human serum albumin (HSA). Radioactivity of standards and cell pellets were measured with a calibrated well-type γ-counter (LKB 1282; CompuGamma). The IRF was determined for each separate experiment by extrapolating to conditions representing infinite antigen excess, corrected for non-specific binding.

In vitro evaluation of ^{89}Zr-AMG 110.

Internalization of ^{89}Zr-AMG 110 was determined by incubating 10^6 HT-29 cells with 50 ng ^{89}Zr-AMG 110 for 1 h at 4°C. Subsequently, unbound ^{89}Zr-AMG 110 was removed by rinsing with PBS containing 1% HSA. Remaining activity, defined as initial cell associated radioactivity, was measured in a calibrated well-type γ-counter and set to 100%. Next cells were resuspended in culture medium (RPMI + 10% FCS, including T = 0) and incubated 1, 2 or 4 h at 4°C or 37°C. Thereafter, medium was removed and cell pellet activity, defined as membrane bound + internalized, was measured in a calibrated well-type γ-counter. Finally, cells were stripped using urea buffer (4 M urea, 2 M glycine, pH 2.0) *(5)* and washed twice with urea buffer. Radioactivity in the stripped pellet, representing the internalized radioactivity, was measured in a calibrated well-type γ-counter. Internalization was determined by the following formula: (internalized radioactivity/initial cell associated radioactivity) x 100%.

Flow cytometry.

To determine EpCAM expression by the cell lines, flow cytometry was performed with a BD Accuri™ C6 flow cytometer (BD Biosciences). HT-29, FaDu or HL-60 cells were incubated for 1 h at 4 °C, with 20 μg/mL mouse anti-human EpCAM antibody (Abcam, ab20160) and washed twice using phosphate buffered saline (PBS; 140 mmol/L NaCl, 9 mmol/L Na_2HPO_4, 1.3 mmol/L NaH_2PO_4; pH = 7.4, UMCG) containing 0.5% FCS and 2 mM ethylenediaminetetraacetic acid. Subsequently, cells were incubated for 1 h at 4°C with (0.01 mg/mL) goat anti-mouse phycoerythrin secondary antibody (Southern Biotech). Cells were finally washed twice and EpCAM expression was assessed. Membrane expression was calculated as mean fluorescent intensity and expressed as percentage of HT-29 signal.

In vivo fluorescent imaging

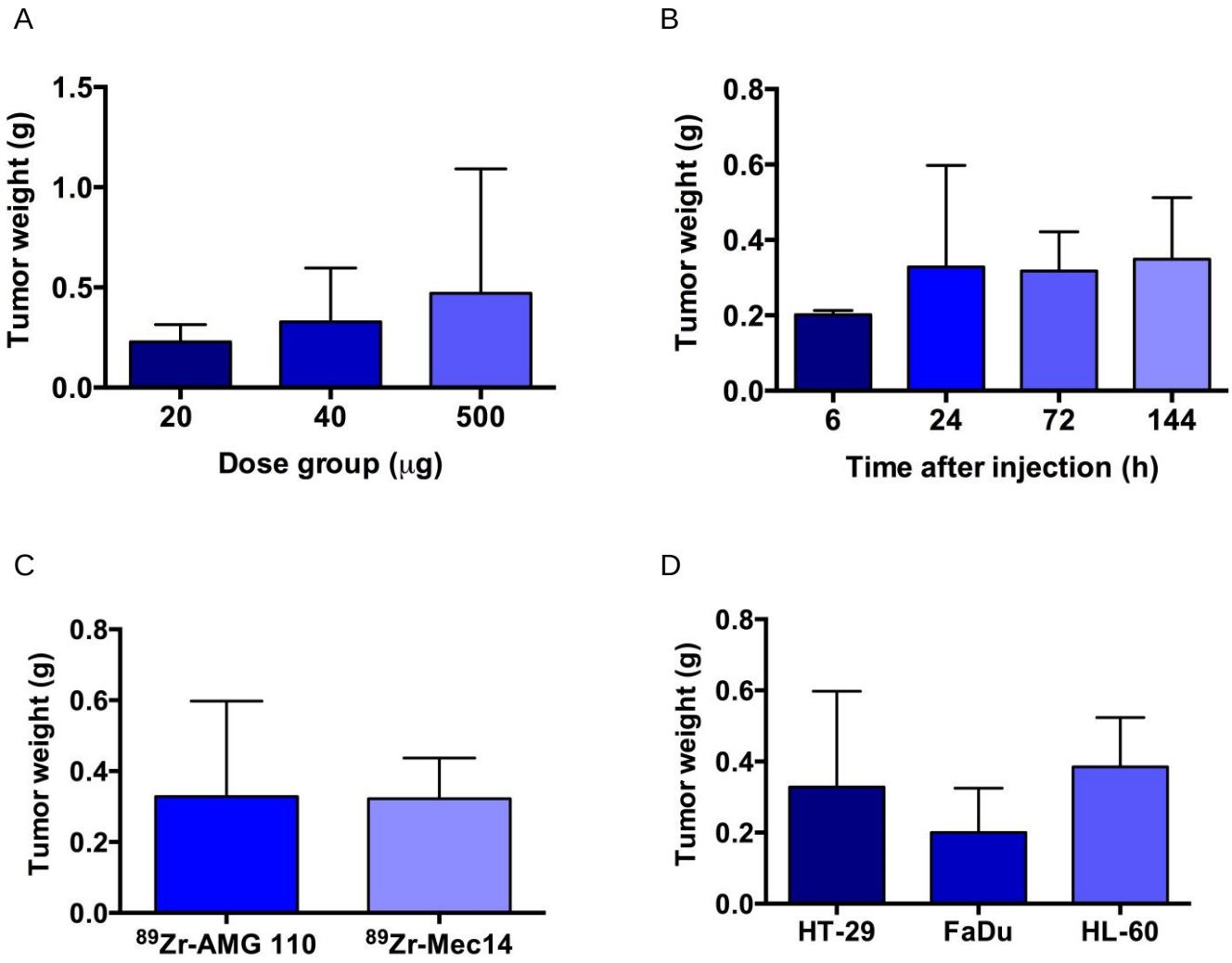
For NIR fluorescence imaging, mice bearing HT-29 xenografts (n = 4) were co-injected with 40 μg 800CW-AMG 110 and 40 μg 680RD-Mec14. Mice undergoing fluorescent imaging were kept on an alfalfa-free diet to minimize autofluorescence. Imaging was performed at 0.5, 1, 3, 6 and 24 h after tracer injection, using the IVIS Spectrum (Caliper Life Sciences) imaging system. Excitation wavelengths were set at 640 nm for 680RD-Mec14 and 745 nm for 800CW-AMG 110. Data were analyzed using Living Image 3.2 software (Caliper Life Sciences). Tumor signal was determined by drawing regions of interest around tumor boundaries for both 680RD-Mec14 and 800CW-AMG 110.

RESULTS

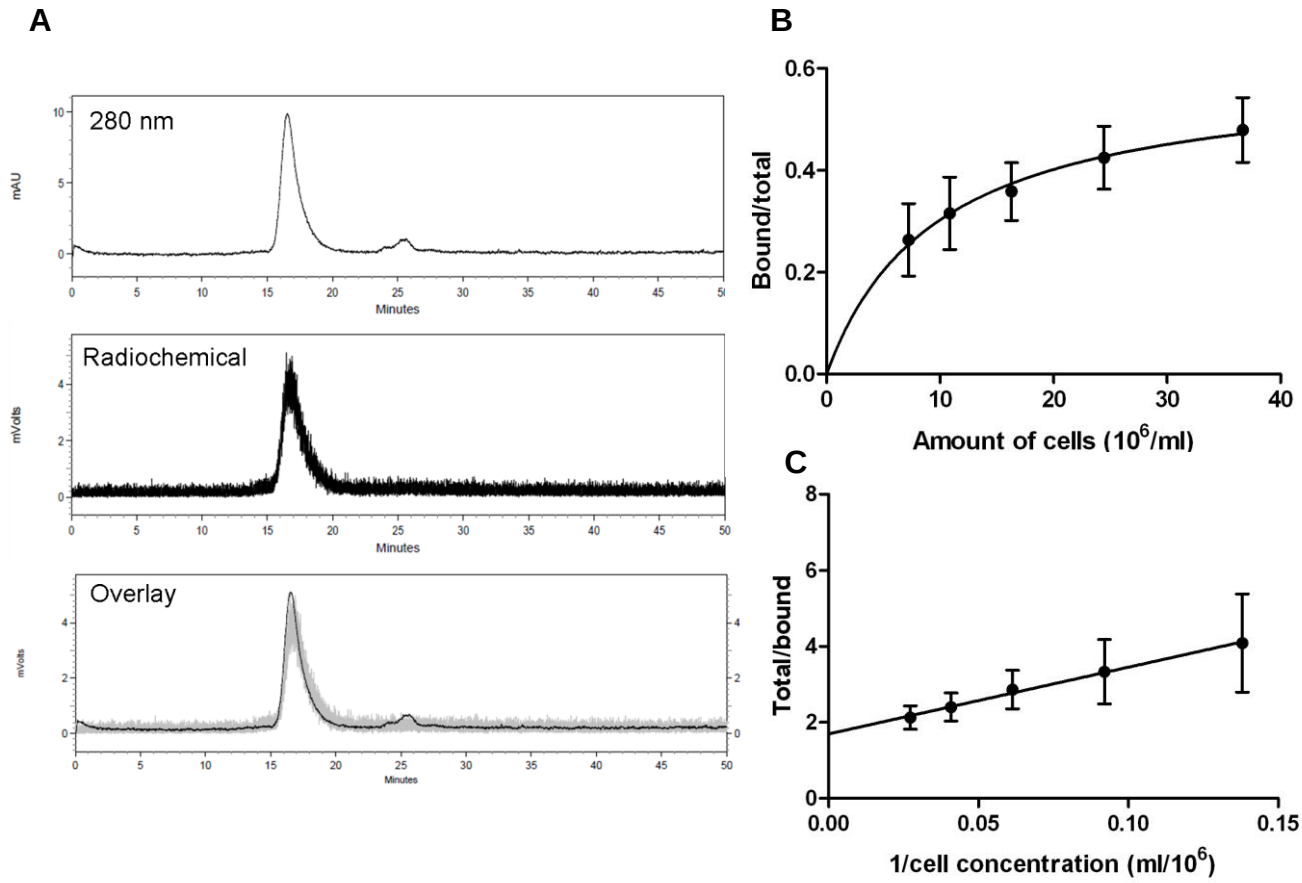
Labeling and quality control of ^{89}Zr-AMG 110 and ^{89}Zr-Mec14.

AMG 110 and Mec14 were successfully conjugated with N-sucDf-TFP (ratio 1:3) and labeled with ^{89}Zr. The retention time for AMG 110 and Mec14 was approximately 17 min. ^{89}Zr-N-sucDf-TFP, low-molecular-weight impurities and buffer additives eluted around 25 min on the SE-HPLC (Supplemental Fig 2A). Upon N-sucDf-TFP conjugation and ^{89}Zr labeling of AMG 110 and Mec14, SE-HPLC did not show aggregation or fragmentation and radiochemical purity was confirmed using trichloroacetic acid precipitation tests (respectively 96.8 ± 1.1% and 96.2%; n = 10 and n = 1).

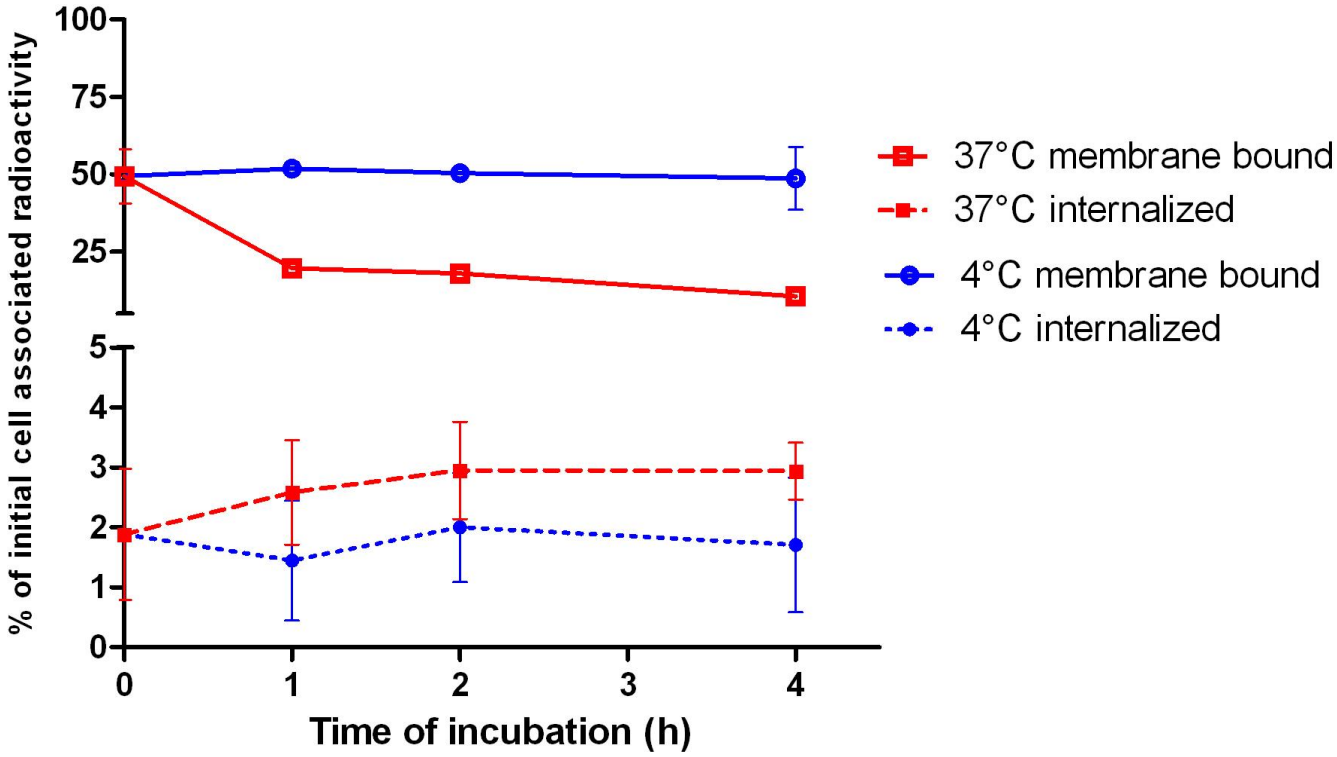
Conjugation of N-sucDf-TFP to AMG 110 and subsequent labeling with ^{89}Zr resulted in a mean IRF of 0.60 ± 0.03 for EpCAM (Supplemental Figs. 2B and 2C; n = 6). Subsequent to EpCAM binding, ^{89}Zr-AMG 110 showed minimal internalization by HT-29 cells at 37°C *in vitro* (Supplemental Fig. 3).



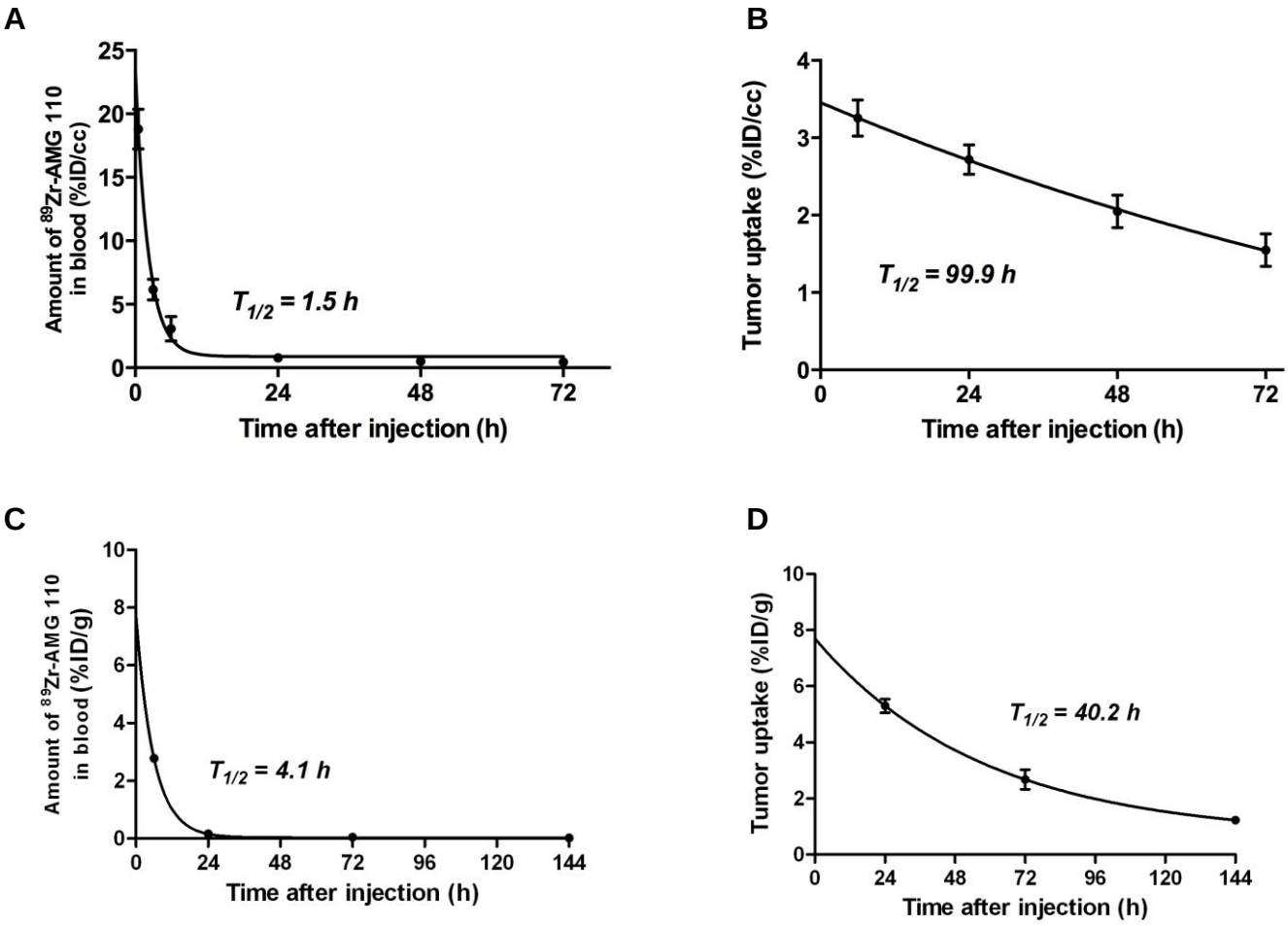
SUPPLEMENTAL FIGURE 1. Differences in ex vivo tumor weights between the experiments, as measured after mice were sacrificed. (A) Difference in tumor weights after resection from mice injected with ^{89}Zr-AMG 110 at 20 µg (n = 5), 40 µg (n = 4) or 500 µg (n = 3) dose levels. (B) Difference in tumor weights at 6 (n = 3), 24 (n = 4), 72 (n = 4) and 144 h (n = 5) after injection of 40 µg ^{89}Zr-AMG 110. (C) Difference in tumor weights at 24 h after injection of 40 µg ^{89}Zr-AMG 110 (n = 4) or 40 µg ^{89}Zr-Mec 14 (n = 6). (D) Difference in tumor weights between HT-29 (n = 4), FaDu (n = 5) and HL-60 (n = 6) tumors at 24 h after injection of 40 µg ^{89}Zr-AMG 110.



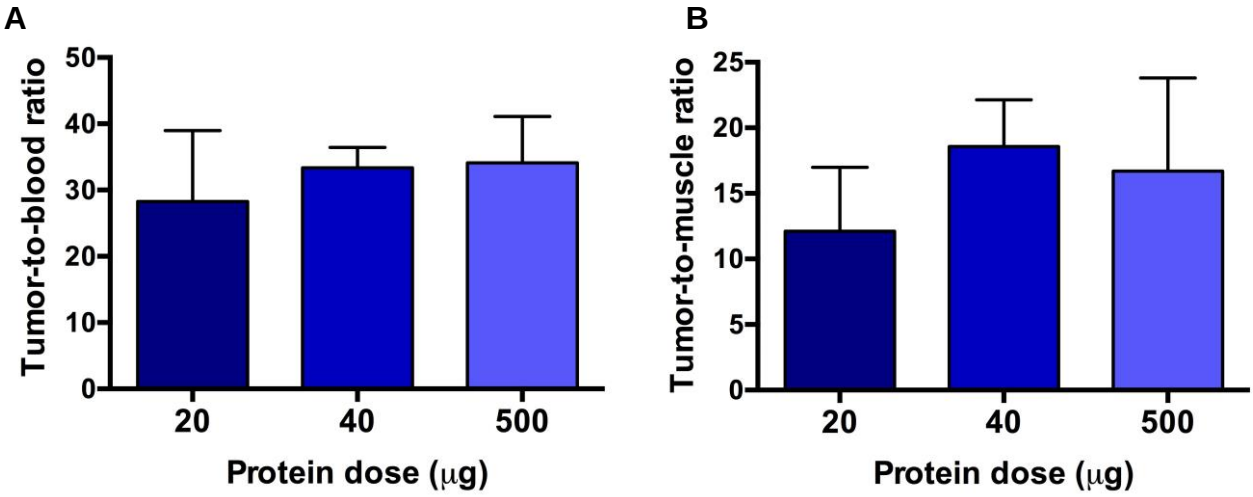
SUPPLEMENTAL FIGURE 2. Quality control of ^{89}Zr-AMG 110 and ^{89}Zr-Mec 14. Panel A shows a typical SE-HPLC chromatogram of ^{89}Zr-AMG 110 (upper panel) or ^{89}Zr-Mec 14 (lower panel), which is an overlay of 280 nm and radiochemical signal. The binding of ^{89}Zr-AMG 110 to an increasing number of HT-29 cells is shown in panel B. Its reciprocal plot to infinite antigen excess was used to determine immunoreactive fraction. Data is presented as mean ± SD.



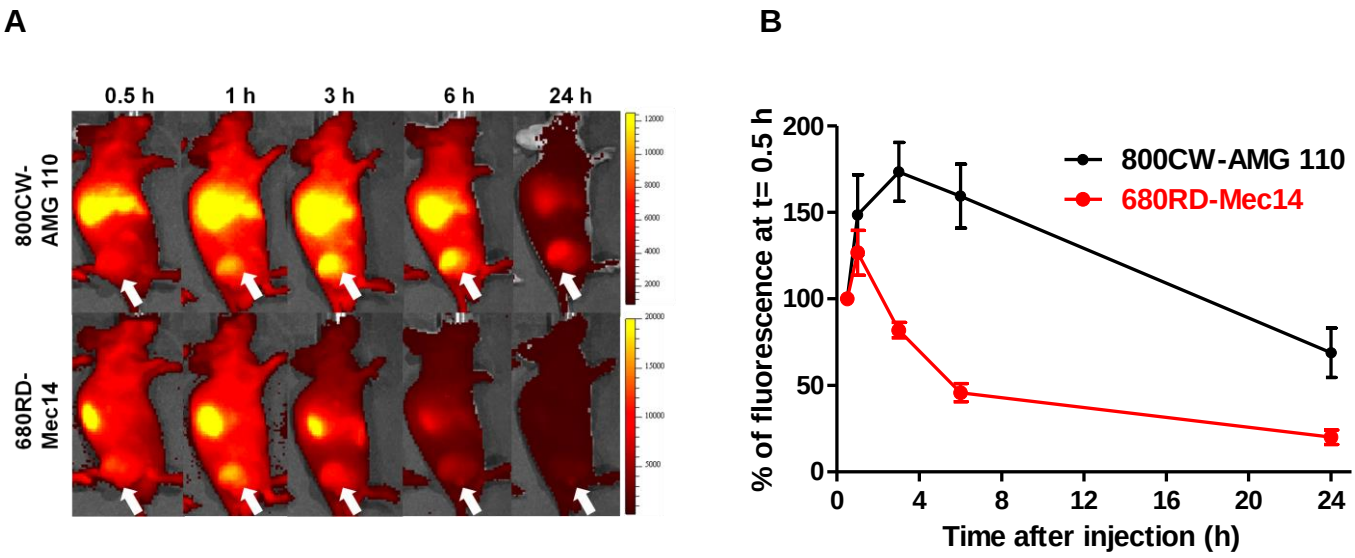
SUPPLEMENTAL FIGURE 3. Membrane binding and internalization of ^{89}Zr-AMG 110 after binding EpCAM on HT-29 cells. Membrane bound and internalized fraction is expressed as percentage of initial cell associated radioactivity. Data is presented as mean ± SD. At several time points in the graphs of membrane bound ^{89}Zr-AMG 110, SD is not visible due its small size.



SUPPLEMENTAL FIGURE 4. Biological half-life of ^{89}Zr-AMG 110 in blood and tumor. Biological half-life of 20 µg ^{89}Zr-AMG 110 in blood (A) and tumor (B), based on %ID/cc. Biological half-life of 40 µg ^{89}Zr-AMG 110 in blood (C) and tumor (D), based on %ID/g. Data is presented as mean ± SD.



SUPPLEMENTAL FIGURE 5. Dose dependent tumor-to-blood (A) and tumor-to-muscle ratios (B). Mice were injected with 20 µg (n = 5), 40 µg (n = 4) or 500 µg (n = 3) protein doses. No significant differences were observed in blood or tissue levels between the dose groups. Data is presented as mean ± SD.



SUPPLEMENTAL FIGURE 6. Representative sagittal two-dimensional *in vivo* fluorescence images at indicated time points, after coinjection of 40 µg 800CW-AMG 110 and 40 µg 680RD-Mec14. White arrow indicates location of the tumor. (A). Fluorescent tracer uptake in HT-29 tumors over time, normalized to absolute fluorescence levels at 30 min after tracer injection (B). Data is presented as mean ± SD.

REFERENCES

1. Verel I, Visser GW, Boellaard R, Stigter-van Walsum M, Snow GB, van Dongen GA. ^{89}Zr immuno-PET: Comprehensive procedures for the production of ^{89}Zr-labeled monoclonal antibodies. *J Nucl Med*. 2003;44:1271-1281.
2. Terwisscha van Scheltinga AG, van Dam GM, Nagengast WB, et al. Intraoperative near-infrared fluorescence tumor imaging with vascular endothelial growth factor and human epidermal growth factor receptor 2 targeting antibodies. *J Nucl Med*. 2011;52:1778-1785.
3. Nagengast WB, de Vries EG, Hospers GA, et al. In vivo VEGF imaging with radiolabeled bevacizumab in a human ovarian tumor xenograft. *J Nucl Med*. 2007;48:1313-1319.
4. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Methods*. 1984;72:77-89.
5. Oude Munnink TH, de Vries EG, Vedelaar SR, et al. Lapatinib and 17AAG reduce ^{89}Zr-trastuzumab-F(ab')$_2$ uptake in SKBR3 tumor xenografts. *Mol Pharm*. 2012;9:2995-3002.