SUPPLEMENTAL MATERIALS.

Supplemental Material S1. *Detailed methods:*

Synthesis of DTPA bis-tyramide. One mmol DTPA-bis (anhydride) was reacted with 2.2 mmol tyramine in the presence of triethylamine in dry dimethylformamide for 48h at room temperature followed by purification by repeated acetone precipitation from 50% methanol. The resultant compound was analyzed using ^{1}H NMR and mass-spectrometry (FAB-MS: found: 632 $[M+H]^{+}$, theoretical m/e, 631.29). The gadolinium salt of DTPA bis-tyramide (diTyr-DTPA(Gd) was prepared by dissolving 0.1 mmol of DTPA bis-tyramide in 0.5M trimethylammonium citrate (pH 6) and adding 1.05 eq. $GdCl_3$ hexahydrate under argon in the dark for 72 h at room temperature. The solution was dried in a vacuum, solubilized in water, and purified using reversed-phase HPLC.

Synthesis of monoclonal antibody conjugates. Conjugates were synthesized and purified as described in (1). Humanized anti-EGFR EMD72000 (humanized mAb 425 (2)) was from Merck KGaA, Darmstadt, Germany, anti-EpCAM (AUA1) was from AbCam (Cambridge MA). Antibodies were dialyzed against 10 mM PBS, pH 7.5 before use. EMD72000 (60 μM in 0.1M sodium bicarbonate pH 8) was covalently modified using SANH (Thermo-Fisher Corp.) in DMF (final concentration - 300μM) for 30 min at room temperature. The modified antibody was purified on Sephadex G25m spin-columns (PD10, GE Healthcare BioSciences Corp., Piscataway NJ) equilibrated with 0.1 M sodium acetate, pH 4.9. Recombinant glucose oxidase (GOX) from *Aspergillus niger* (EMD Merck-Calbiochem), 120 μM in 0.1 M sodium bicarbonate pH 8, (25 nmol), was modified by adding 100 nmol C6-SFB (Thermo-Fisher Corp.). After 30 min incubation, GOX-formyl benzoate was purified as described above. Horseradish peroxidase (100 nmol, Type IX, Sigma) in 0.25 ml of 0.1 M sodium acetate, pH 5 was oxidized using 10-molar excess of sodium periodate for 30 min. The reaction was stopped by adding 0.1 M glycerol (final concentration) followed by purification in Sephadex G25m spin-columns. The oxidized peroxidase was incubated in the presence of 0.1 M hydroxylamine for 3 h and purified using

Sephadex G25m chromatography. Conjugation of formylbenzoyl groups to peroxidase was performed by using a 10-fold molar excess of C6-SFB. The number of covalently conjugated 4-formylbenzoyl groups was determined by spectrophotometry at 350 nm (ε =18000 M^{-1} cm). Conjugated 4-hydraziniumnicotinate was determined by using 4-nitrobenzaldehyde and measuring absorbance at 380 nm (ε =22000 $M^{-1}cm^{-1}$). Protein concentrations were determined by using Micro BCA assay (Pierce). 4-hydraziniumnicotinate-modified antibody (final concentration – 2 mg/ml) was ~~were~~ combined with the modified enzymes at molar ratios of 1:2 (antibody: enzyme) in 0.1 M sodium acetate, 0.1% Tween-20, pH 4.9. The incubation was completed in 4 h. To block the remaining aldehyde groups, 2-hydrazinopyridine was added at a final concentration of 0.1 mM. The reaction mixture was separated using Superdex 200 columns (GE-Healthcare Life Sciences, Piscataway NJ) in 0.1 M ammonium acetate, pH 7.0. The peaks that eluted before 200 kD protein and contained peroxidase or glucose oxidase activity were pooled separately, concentrated and washed with PBS using Ultracel-50 membrane concentrators (Millipore, Billerica MA). Concentrated conjugates were analyzed using 4-15% gradient SDS-PAGE.

In vitro cell culture experiments. Gli36ΔEGFR (3) and wild-type Gli36wt cells (4) were propagated on 10%FCS 90% RPMI1640 in the presence of penicillin/streptomycin and 0.5 μg/ml puromycin (Gli36ΔEGFR). Both cell lines were binding chimeric anti-EGFR antibody (cetuximab, C225) and EMD72000 (Fig 1S, A,B). For cross-titration experiments, cells were plated in 96 wells and used when confluent. Conjugates were cross-tittered using sequential 2x dilutions (8x8 wells) in 2% FCS in DPBS, pH 7.4 and used in the range of 1000 – 7.5 ng total conjugate (i.e., a mixture of mAb-HRP and mAb-GOX) per well. Cells were incubated with conjugates for 30 min, washed and peroxidase activity associated with the cells was determined at 405 nm using 2 mM ABTS in 5 mM glucose in 50 mM sodium citrate-phosphate buffer, pH 5.7. In some experiments, 2 μg of free EMD72000 per well was used as a competitive inhibitor. Titration of conjugate mixtures in Gli36ΔEGFR culture was performed by using dilutions of

mAb-HRP/mAb-GOX mixture at 1:2 (w/w ratio). Measurements of longitudinal relaxation time (T_1) changes were performed on Bruker Minispec mq20 by using reaction mixtures containing 0.05-0.75 mM diTyr-GdDTPA, 5mM glucose, PBS, pH 7.4 in the presence or in the absence of 1 μg conjugate mixture. Cell viability was determined using the XTT technique. Titration data fitting and analysis were performed by using GraphPad's Prism4 software (La Jolla, CA).

Internalization experiments were performed by using mAb-HRP/mAb-GOX conjugate mixture at 1:2 (w/w ratio). Adherent cells in 6-well plates (4 million cells/well) were incubated with conjugate mixtures either at 4°C or at 37°C. The surface bound conjugates were eluted with 0.5 ml cold 0.2M glycine, pH 2.5 for 15 min. The eluate was immediately neutralized with 1M Tris pH 8.0. To extract internalized conjugates 0.5 ml of 1.0% Igepal CA-630 in the presence of protease inhibitors was added to each well and plates incubated for 15 min. The amount of bound and internalized conjugates was determined by measuring the initial rates of HRP/GOX-coupled ABTS oxidation by adding of 5 mM ABTS, 5mM glucose (final concentrations) in sodium citrate, pH 5.5 to the sample aliquots and measuring absorbance at 405 nm over time. The serially diluted conjugate mixture at a constant mAb-HRP:mAb-GOX ratio and known concentrations was used for calibration.

Membrane proteins extraction from the cells. Membrane proteins from Gli36ΔEGFR and Gli36WT cells were extracted using CNM compartmental protein extraction kit (BioChain Institute Inc., Hayward, CA) following manufacturer's recommendations. Cells were harvested using TrypLE Express cell detachment solution (Invitrogen Corp. Carlsbad, CA) and washed with PBS buffer. The cells were counted, centrifuged at 1000 RPM for 5 min and supernatant was removed. A cocktail of protease inhibitors was added to extraction buffers at the recommended concentration before protein extraction. All extraction steps were performed at 4°C.

To the cell pellet ice cold buffer C (2 ml/ $2 \cdot 10^7$ cells) was added. The cells were suspended and rotated using a Labquake rotating Micro-Tube mixer for 20

min. The cell mixture was passed ~75 times through a 3 ml syringe fitted with a 28 gauge needle base after removing the needle tip to disrupt the cells. The cell suspension was centrifuged at 15,000 g for 20 min.

The supernatant containing cytoplasmic proteins was transferred to a new tube and saved. The remaining pellet was washed by suspending in ice cold buffer W (4.0 ml per 20 million cells), rotated for 5 min and centrifuged at 15,000 g for 20 min. The supernatant was discarded and the cell pellet was resuspended in ice cold buffer N at 1.0 ml per 20 million cells and rotated for 20 minutes. Nuclear proteins were recovered by centrifugation at 15,000 g for 20 min. To the remaining pellet, ice cold buffer M at 1.0 ml per 20 million cells was added and the tube rotated for 20 min. Cells were spun at 15,000 g for 20 min to recover the membrane proteins in the supernatant. The protein concentration was measured in all the fractions by Micro BCA Protein Assay kit (Thermo Scientific). Fractions were aliquoted and stored at –70°C.

Supplemental Material S2*. Evaluation of the temporal evolution of tumor signal decay in vivo following infusion of diTyr-GdDTPA*: The temporal evolution of the signal decay in the tumor following infusion of diTyr-GdDTPA was evaluated separately for each of the two groups (Day 1 and Day 2). Depending on the size of the tumor, one to four slices were selected covering the central core region of the tumor at each time-point. Using the ImageJ software package (5), a region-of-interest (ROI) was carefully drawn to delineate the boundary of the contrast-enhanced region for each slice. ROIs were also drawn to distinguish the tumor interface and tumor core regions (Fig. 2S). The whole ROI was drawn to circumscribe the entire contrast-enhanced region of the tumor visible at the first time-point collected after the contrast injection. The core ROI was drawn using a later time-point that better depicted the outline of the core region due to the faster signal decay in the core as compared to the interface. The interface ROI was drawn by tracing over the region defined by the difference between the whole ROI and the core ROI. The mean signal intensity values within each of the ROIs were then calculated using ImageJ.

For each animal, the signal intensity data for the core and interface ROIs from each relevant tumor slice was plotted to generate a separate time-series plot for Day 1 (no conjugates) and Day 2 (with conjugates). Due to the heterogeneity of tumor growth and variability in the exact dose of contrast delivered from one animal to another, each signal-intensity time-point was normalized relative to the pre-contrast time-point for each slice:

$$S_N(t) = \frac{S_{post}(t) - S_{pre}(0)}{S_{pre}(0)} \times 100 \qquad \text{[Eq. 1]}$$

where $S_N(t)$ is the normalized signal intensity at time t, $S_{post}(t)$ is the signal intensity at time t, and $S_{pre}(0)$ is the signal intensity in the pre-contrast slice.

For each animal, the normalized signal-decay plots for each brain slice from Day 1 and Day 2 were used to calculate signal-decay time constants using a monoexponential and a biexponential model:

$$y_{monoexp}(t) = A_0 \cdot e^{-t/\tau_0} + offset \qquad \text{[Eq. 2]}$$

$$y_{biexp}(t) = A_1 \cdot e^{-t/\tau_1} + A_2 \cdot e^{-t/\tau_2} \qquad \text{[Eq. 3]}$$

where $y_{monoexp}(t)$ and $y_{biexp}(t)$ are the signal intensities at time t; A_0, A_1, and A_2 are the amplitudes of each signal-decay component; τ_0, τ_1, and τ_2 are the signal-decay time constants for the respective models.

For each animal, the normalized signal-decay plots of the core and interface ROIs for each brain slice from Day 1 and Day 2 were fitted with both models. Mean values for τ_0, τ_1, and τ_2 were obtained for the core ROI and the interface ROI for each animal by averaging the respective signal-decay time constants of each ROI from all relevant tumor slices of the same animal. The mean values for τ_0, τ_1, and τ_2 were calculated for each animal on Day 1 and on Day 2. Student's paired t test was performed on the overall average of the signal-decay time constants from all the animals to check for any significant differences either between the ROIs or between Day 1 and Day 2.

To determine which model (monoexponential or biexponential) better represented the normalized signal-decay data, the χ^2 statistics for the monoexponential ($\chi^2_{monoexp}$) and biexponential (χ^2_{biexp}) models were compared using an F test based on a variant of the reduced χ^2-squares ratio:

$$F_{(\upsilon_{monoexp}-\upsilon_{biexp},\upsilon_{biexp})} = \frac{\left(\chi^2_{monoexp} - \chi^2_{biexp}\right)/\left(\upsilon_{monoexp} - \upsilon_{biexp}\right)}{\chi^2_{biexp}/\upsilon_{biexp}} \qquad \text{[Eq. 4]}$$

where $\upsilon_{monoexp}$ and υ_{biexp} are the number of degrees of freedom corresponding to $\chi^2_{monoexp}$ and χ^2_{biexp} for the monoexponential and biexponential model, respectively (6). For this study, the F statistic ratio simplifies to:

$$F_{(1,N-4)} = \frac{\left(\chi^2_{mono\,exp} - \chi^2_{bi\,exp}\right)}{\chi^2_{bi\,exp} / (N-4)} \qquad \text{[Eq. 5]}$$

where N is the number of data points. This ratio measures how much the additional exponential term in the biexponential model improves the reduced χ^2 by taking into account the number of parameters in the fitting equation (7). This calculated *F* statistic was compared to tabulated values of the *F* statistic with 1 and $N - 4$ degrees of freedom. If the *F* statistic was greater than the tabulated *F* value for the desired statistical confidence, then the biexponential model was accepted as the better fitting model for the data. If the *F* statistic was less than the tabulated *F* value, then the monoexponential model was chosen for fitting the data. *P* values less than 0.05 were considered statistically significant.

Supplemental Material S3. *Derivation of relationship between decay time constant and EES volume using the Tofts and Kermode model:* The decay time constants (DTCs) of diTyr-GdDTPA derived from the temporal decay of the normalized signal intensities were used as the basis for comparing the contrast agent (CA) kinetics between individual animals. This approach is analogous to that employed in dynamic contrast-enhanced MRI (DCE-MRI) (8) except that only the washout portion of the kinetic curve was measured in our study. Although the kinetic parameters calculated in this study differ from those used in the model of Tofts and Kermode (8), the parameters still relate to tumor perfusion, vascular permeability, and the volume of the extravascular extracellular space (EES).

The short DTCs (τ_2) for free diTyr-GdDTPA following conjugate pre-treatment (Day 2) was significantly shorter than the corresponding diTyr-GdDTPA DTCs (τ_0) without conjugate pre-treatment (Day 1). The reduced DTCs for free diTyr-GdDTPA following conjugate pre-treatment may arise from a decrease in vascular permeability which could occur due to a reduction in the volume of the EES (derivation shown below). This reduction in volume might arise due to a "binding site barrier" effect (9), which would imply that successful binding of the anti-EGFR conjugates impedes the penetration of unbound conjugates, thus leaving less EES volume available for the CA to permeate into from the blood plasma (9, 10). Using the Tofts and Kermode model, we will establish that a reduction in the DTC is proportional to a reduction in the EES volume fraction.

The Tofts and Kermode model derives the lesion curve $C_1(t)$ by solving a first-order linear differential equation (Eq. A9 in (8)), which has the following solution (Eq. A10 in (8)):

$$C_1(t) = D\left(\frac{m_3 a_1}{m_3 - m_1} e^{-m_1 t} + \frac{m_3 a_2}{m_3 - m_2} e^{-m_2 t} + c e^{-m_3 t}\right) \qquad \text{[Eq. 6]}$$

where a_1, a_2, m_1, and m_2 are parameters that are calculated from the plasma curve $C_p(t)$ (Eq. 1 in (8) D is the contrast dose administered, c is a constant that is calculated from the initial condition $C_1(0_+)=0$, and m_3 is the variable that is calculated from applying this model to a lesion curve data set with a wash-in and wash-out phases. The parameter m_3 is defined as the exchange rate constant between EES and blood plasma (k_{ep}) as defined by Tofts *et. al.* (11):

$$k_{ep} = K^{trans} / \upsilon_e = 1/\tau_c \qquad \text{[Eq. 7]}$$

where K^{trans} is the volume transfer constant between blood plasma and the EES, υ_e is the volume fraction of the EES, and τ_c is the DTC. In our study we

calculated the DTC, which is inversely proportional to k_{ep}. We can establish from this relationship that τ_c is directly proportional to υ_e. Thus, a decrease of the DTC would suggest that there is a decrease of EES volume fraction. K^{trans} can also be reduced (due to a decrease in transendothelial permeability in the presence of conjugates) since it is equal to the permeability surface area product between blood plasma and the EES per unit volume of tissue (11). However, the decrease in υ_e is greater than the decrease of K^{trans}, which is supported by the fact that we observed a decrease of diTyr-GdDTPA DTCs on Day 2, i.e. after the pre-injection of conjugates.

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