Supplementary Experimental Procedures

Cell lines

For immunization and specificity analyses, a panel of cell lines (parental or transfected with either the human EGFR gene or the EGFRvIII gene) were used, including murine fibroblast cell lines NIH-3T3, NIH3T3-EGFRvIIIex (NIH-3T3 transfected with EGFRvIII extracelluar domain expression on the cell surface), human glioblastoma cell lines U87 MG (expressing low levels of endogenous wtEGFR), U87 MG-EGFRvIII (U87 MG cells with forced EGFRvIII expression) and human squamous cell carcinoma cell line A431 (expressing high levels of wtEGFR). All cell lines were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The dihydrofolate reductase (DHFR)-deficient CHO cell line DG44 (Invitrogen, Carlsbad, CA) was cultured in IMDM supplemented with 7% fetal bovine serum and hypoxanthine– thymidine (Invitrogen, Carlsbad, CA).

Immunization of mice

1×10^6 mouse embryonic fibroblast cell line NIH/3T3-EGFRvIIIex was used to immunize the mice in the first time. After 4 weeks, the BALB/c mice were boost with the recombinant EGFRvIIIex protein by s.c. injection. Then, the BALB/c mice were immunized with NIH/3T3-EGFRvIIIex cells or recombinant EGFRvIIIex protein by 2 week intervals for another four times. Complete Freund's adjuvant (Sigma) was used for the first injection, and incomplete Freund's adjuvant (Sigma) was used for subsequent injections. Blood samples were collected from the mice 2 weeks after each injection and an ELISA test for the detection of antibody was performed on their sera.

Cell fusion and selection of hybridoma clones

One week before cell fusion, 3×10^6 NIH/3T3-EGFRvIIIex cells were injected intrasplenically into the mice. The mice were sacrificed and their spleen mononuclear cells were separated under sterile conditions. Mouse spleen lymphocytes and myeloma cells (SP2/0 cell line from ATCC), were washed 3–4 times separately using RPMI medium (Gibco). The spleen lymphocytes and myeloma cells were then mixed and fused together by stepwise addition of 50% polyethylenglycol (PEG) solution (Sigma). Then, the cells were seeded in 96 wells tissue culture plates in HAT (Hypoxantine, Aminopterin, thymidine) (Gibco) containing RPMI supplemented with 10% FCS and incubated at 37°C and 5% CO_2. After 3~4 days, the cells were added 100µl of HT (Hypoxantine, Thymidine) (Gibco) containing RPMI medium. The hybridoma clones were formed after about 2 weeks and their supernatants were analyzed by ELISA test.

ELISA assay

1×10^4 NIH/3T3-EGFRvIIIex cells were plated in 96-well plates and incubated at 37°C. Recombinant EGFRvIIIex protein (50 ng) was coated on an ELISA plate and incubated overnight at 4°C. The next day, the plates were washed three times with PBS containing 0.05% Tween 20. Serial dilutions of mice sera (or supernatant of cell cultures) (50 µl) were added to each well and incubated for 2 hours at 37°C. The plates were washed as described previously. HRP conjugated goat anti-mouse immunoglobulin (50 µl) (Kang-Chen Bio-tech, Shanghai, China) (diluted 1/1000

in PBS-T) was added to the plates and incubated for 2 hours at 37°C before washing. The wells were then washed three times in washing buffer before adding 100 µl of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline sulfonic acid) substrate solution (1 mM ABTS, 29 mM anhydrous citric acid, 41 mM dibasic Na phosphate, pH 4.2, 0.03% H2O2) to each well. The plates were incubated at 37°C for 15 min and absorbance at 410 nm was recorded using an ELISA reader (Bio-Rad)

Production of ascetic fluid and antibody purification

For in vivo production of antibodies, paraffin solution (0.2 ml) (Sigma) was injected into the peritoneum of BALB/c mice. We injected 1×10^6 hybridoma cells the following week and a few days after tumor formation. The peritoneal fluids were extracted by syringe, centrifuged and frozen until use. Antibody was purified on a protein G column (GE Healthcare Bio-Sciences).

FACS analysis of 12H23 binding activity

Cells (1×10^6) were collected by centrifugation and incubated with 20 µg/ml primary antibody in phosphate-buffered saline containing 1% newborn calf serum for 45 min at 4°C. After being washed with cold phosphate-buffered saline containing 1% newborn calf serum, the cells were incubated for an additional 45 min at 4 °C with a fluorescein (FITC)-conjugated goat anti-mouse antibody (Kang-Chen Bio-tech, Shanghai, China) in the dark. For each sample, at least 10,000 cells were analyzed by FACS cytometry (Beckman Coulter Epics Altra, Miami, FL) and MultiCycle AV for Windows 5.0 (Phoenix Flow Systems, San Diego, CA).

Treatment of Huh7-EGFRvIII xenografts in an established nude mice model with C225 and 12H23.

Huh7-EGFRvIII cells (3×10^6) were subcutaneously injected into 4- to 6-week-old nude mice when tumors had reached a mean tumor volume of 200 mm^3. The mice were then randomly allocated into three groups (n = 6) and treated with (i) vehicle (sterile PBS), (ii) 25 mg/kg C225 in sterile PBS or (iii) 25 mg/kg 12H23 in sterile PBS. Injections were administered intraperitoneally three times per week for 2 weeks. Tumor volumes were measured every other day in two dimensions with Vernier calipers. Tumor volumes were calculated using the formula: (length × $width^2$) × 0.5. Twenty-four days after treatment, the mice were sacrificed by cervical dislocation under anesthesia. The tumors were then surgically excised and weighed. The data are expressed as the percentage of tumor growth inhibition.

Identification of the epitope for 12H23

The EGFR extracellular S1 (EGFR, cysteine rich region, 161G to 313C)domain and S2 (EGFR cysteine rich region, 458G to 614T) domain were recombinantly expressed with N12 (origin from pIII protein of M13 phage) in *E. coli*. EGFR-derived peptide VK21 (^{284}VRACGADSYEMEEDGVRKCKK^{304}), CC16 (^{287}CGADSYEMEEDGVRKC^{302}), CV13 (^{287}CGADSYEMEEDGV^{299}), and DC13 (^{290}DSYEMEEDGVRKC^{302}) were also recombinantly expressed with N12 (origin from pIII protein of M13 phage) as a fusion protein in *E. coli*. All recombinant proteins were purified by an NTA-Ni affinity column (GE Healthcare Bio-Sciences). Then, the EGFR-derived recombinant proteins were coated on an ELISA plate. 12H23 was used as primary antibody. The ELISA assays were performed as described above.

Sequencing of murine 12H23 variable antibody regions

Total RNA was extracted from the 12H23 hybridoma with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RACE (rapid amplification of cDNA ends) was carried out with a 5'-Full RACE Kit (TAKARA, Dalin, China) according to the supplied protocol. Primers for amplification of the heavy and light chains were 5'-end primers, and the sequence was based on the RACE adapt sequence, including 5′RACE Outer Primer CATGGCTACATGCTGACAGCCTA and 5′RACE Inner Primer CGCGGATCCACAGCCTACTGATGATCAGTCGATG. With respect to the 3'-end primers, the light-chain primer hybridized within the mouse kappa constant region, the Outer CK Primerwas ACACGACTGAGGCACCTCCA and the inner CK Primer was TGGATGGTGGGAAGATGGATACA. The heavy-chain primer hybridized within the CHI constant region, the Outer CH Primer was CCAGAGTTCCAGGTCACTGTCACT and Inner CH Primer was CCAGGGTCACCATGGAGTTAGTTT. Outer PCR reactions were carried out with outer primers using the reverse-transcript product as a template. Inner PCR reactions were carried out with inner primers using the outer PCR products as templates. In all cases, 30 cycles of amplification were carried out using the following parameters: denaturation for 1 min at 94°C, annealing for 1 min at 50-55°C and extension for 2 min at 68°C. A final 10-min extension step at 6 °C followed the 30 cycles. The PCR products were analyzed by DNA electrophoresis, and the vH and vK fragments were cloned into the pGEM-TA-Easy vector using the TA Cloning System (Promega, Madison, WI) and then sequenced.

Construction of a mouse–human chimeric antibody

The CH12 antibody was designed to have the variable regions of heavy and light chains of murine 12H23 linked to the human gamma-1 and kappa constant regions, respectively. 12L1 and 12L2 primers containing the restriction enzyme sites *Eco*RV and *Apa*I were used to subclone the 12H23 vL gene into the mammalian cell expression vector PK-DHFR, which already contains the human kappa constant regions. 12H1 and 12H2 primers containing *Eco*RV and *Bsw*I were used to subclone the 12H23 vH gene into the mammalian cell expression vector PH-DHFR, which already contains the human gamma-1 constant regions. The sequences of the primers were as follows: 12L1-ATCGATATCCACCATGGACATGATGGTCCTTGC; 12L2 -CACCGTACG TTTGATTTCCAGTTTGGTGCC; 12H1-ATCGATATCTGCGGCCTAGCTAGCCACCATGAGAGTGCTGATTCTTTTGTGG; 12H2-GATGGGCCCTTGGTGGAGGCTGCAGAGACAGTGACCAGAGTCC.

Expression of CH12 antibody in DHFR-deficient CHO DG44 cells

DNA for transfections was purified from E. coli cells using an AXYGEN Plasmid Midi Kit (Axygen, Hangzhou, China), as recommended by the manufacturer. The plasmids were then digested with restriction enzyme *Pvu*I and purified with an AXYGEN PCR purification Kit (Axygen, Hangzhou, China). For transfection of the DHFR-deficient CHO DG44 cells, plasmids encoding the heavy and light chains of the CH12 antibody were cotransfected into CHO DG44 cells growing at log phase using lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were then plated in 10-cm dishes and cultured with standard medium. After 24 h, the medium was replaced with fresh IMDM medium supplemented with 10% Gibco dialyzed FCS (Invitrogen) and 5 nM methotrexate (Sigma, St. Louis, MO, USA). After 14 days of cell culture, outgrowing

colonies were picked and screened for antibody production. The best producing clones were then subjected to an amplification procedure by gradually increasing the methotrexate concentration up to 400 nM while maintaining all other conditions. The highest producing clones were then used for CH12 antibody production.

Biodistribution of CH12

In the biodistribution experiment, 40 BALB/c nude mice with established Huh7-EGFRvIII xenografts received radiolabelled ^{125}I-CH12 (3 mg. 3.8 mCi ^{125}I-CH12) intravenously via the tail vein (total volume=0.1 ml),.At designated time points after the injection of the radioconjugates (t=4 h, days 1, 2, 3, 5 and 7), groups of mice (n=3) were killed by Ethrane anesthesia. Mice were then exsanguinated by cardiac puncture, and tumours and organs (liver, spleen, kidney, muscle, skin, bone (femur), lungs, heart, stomach, brain, small bowel, tail and colon) were resected immediately. All samples were counted in a dual gamma scintillation counter (Packard Instruments). Triplicate standards prepared from the injected material were counted at each time point with tissue and tumour samples enabling calculations to be corrected for the physical decay of the isotopes. The tissue distribution data were calculated as the mean ± SD of the percent injected dose per gram tissue (%ID g^{-1}) for the CH12 antibody per time point.

Statistical analysis

Data are presented as the mean ± standard error (SE) and were analyzed using Student's t test. Values of $P < 0.05$ were considered statistically significant.

Supplementary Figure Legends

Fig. S1. FACS analysis of 12H23 reactivity with Huh-7 and U87 MG cell lines. Cells were stained with 12H23 as described in "Materials and Methods." 12H23 reacted extensively with EGFRvIII but weakly with wtEGFR. The results were reproduced in three independent experiments.

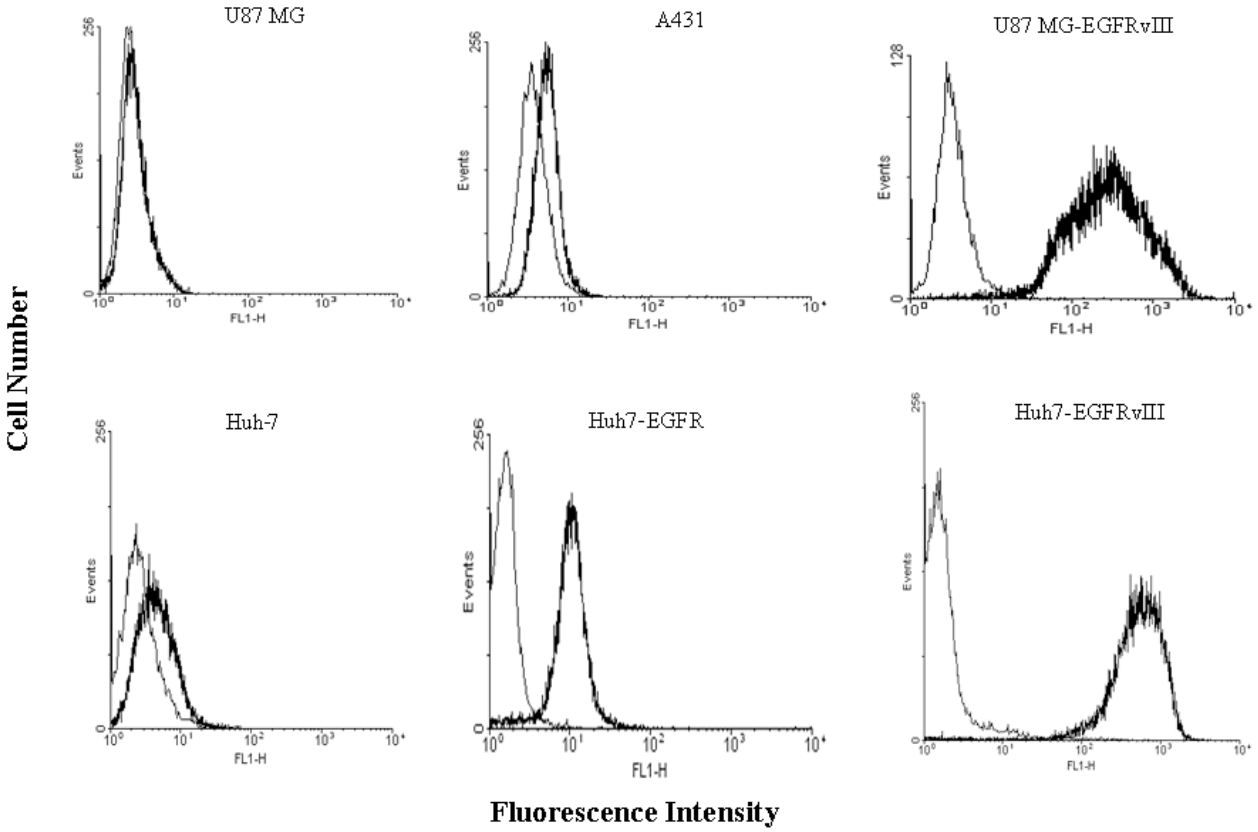
Fig. S2. Antitumor effects of C225 or 12H23 on Huh7-EGFRvIII xenografts *in vivo*. Huh7-EGFRvIII cells (3×10^6) were injected s.c. into 4–6-week-old BALB/c nude mice (n=6). The mice were then injected i.p. with 25 mg/kg C225, 25 mg/kg 12H23 or vehicle (control) when the tumors reached a mean tumor volume of 200 mm^3. Injections were administered three times per week for 2 weeks (bars=SE.). * 12H23 group versus control group for values 27 d after inoculation ($P < 0.05$); ** 12H23 group versus C225 group for values 32 d after inoculation ($P < 0.05$). The inhibitory ratios of C225 and 12H23 for values 32 d after inoculation were 30% and 63.4%, respectively. (A) Data are expressed as the mean tumor volume; (B) the data are expressed as the percentage of tumor growth inhibition.

Fig. S3. Epitope mapping of 12H23. (A) Diagram of EGFR and recombinant EGFR. SG, signal sequence; L1 and L2, large domains; S1 and S2, small cysteine-rich domains; TM, transmembrane segment; TK, tyrosine kinase domain; amino acid residue numbers lie below the structure. N12 is originally from the PIII protein of M13 phage, here acting as a fusion partner. (B) SDS-PAGE of purified EGFR fragments or peptides. (C) Reactivity of mAb 12H23 with fragments of the EGFR

protein using ELISA.

Fig. S4. In vivo biodistribution of iodine-labeled CH12 over 7 days in BALB/c nude mice bearing Huh7-EGFRvIII tumor xenografts (n=3). Results of the biodistribution of ^{125}I-CH12 are presented for each tissue and are expressed as the mean (bars=SD) percent injected dose per gram (%ID g^{-1}).

Figure S1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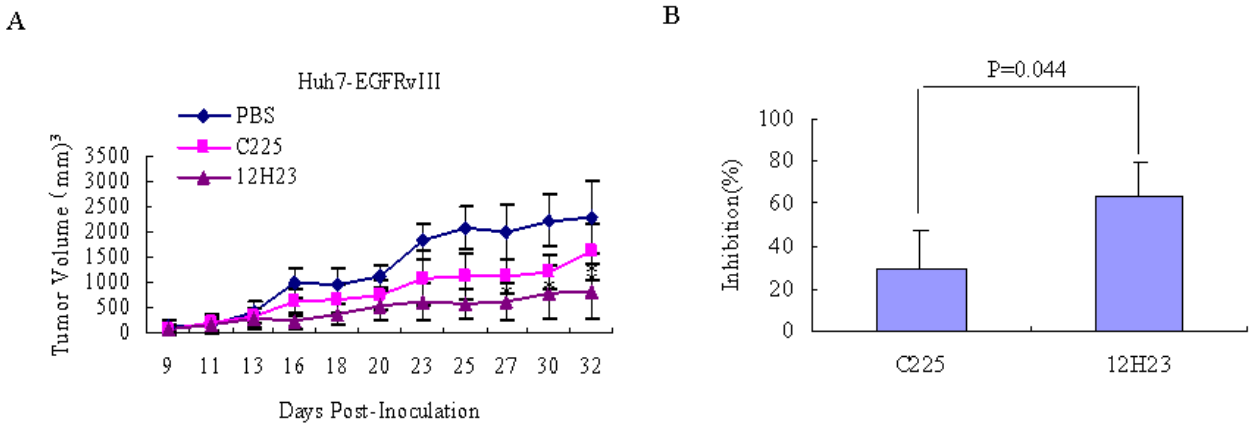
Huh-7
Huh7-EGFR
Huh7-EGFRvIII
Events
FL1-H
Cell Number
Fluorescence Intensity

Figure S2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Huh7-EGFRvIII
PBS
C225
12H23
Tumor Volume (mm)3
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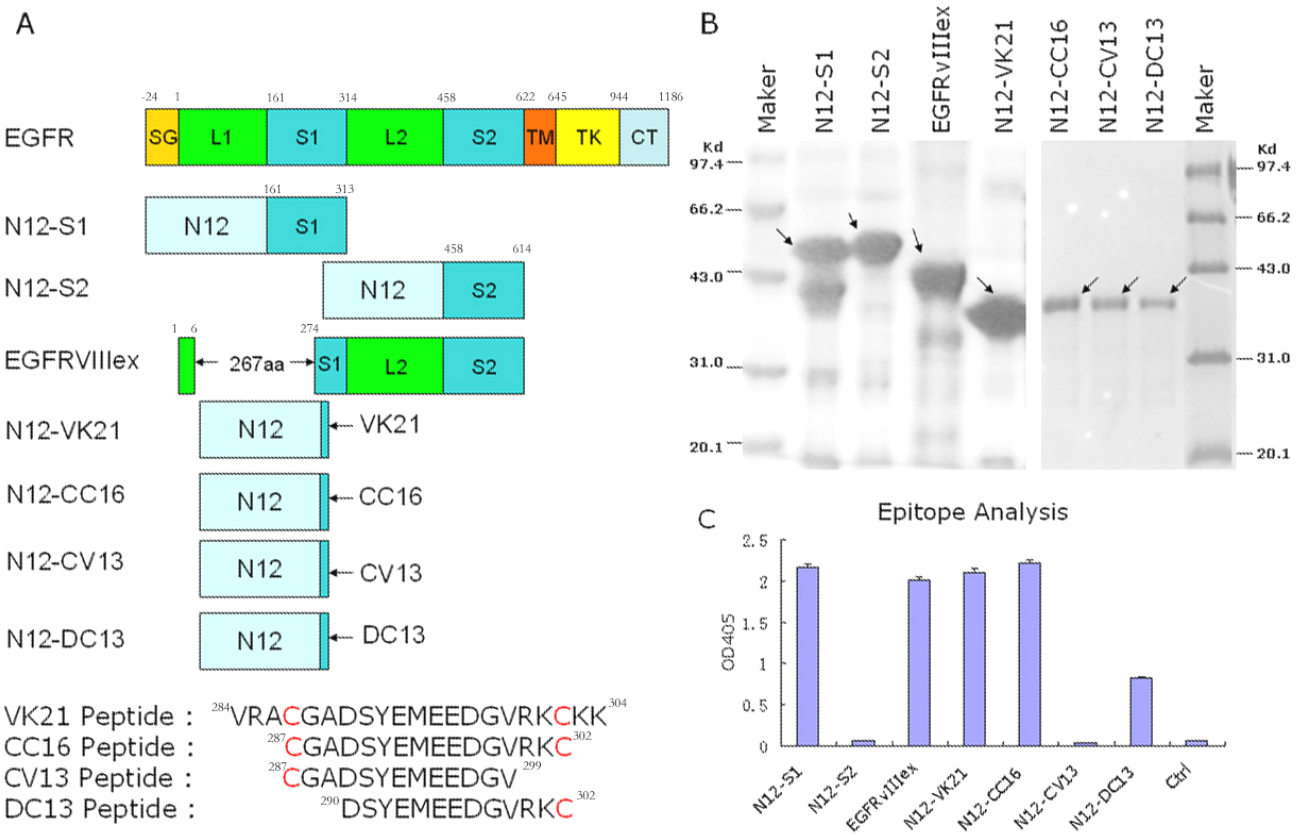
3000
2500
2000
1500
1000
500
0
9 11 13 16 18 20 23 25 27 30 32
Days Post-Inoculation
B
P=0.044
Inhibition(%)
100
80
60
40
20
0
C225
12H23

Figure S3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S1
L2
S2
TM
TK
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N12
S1
N12-S2
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EGFRVIIIex
267aa
S1
L2
S2
N12-VK21
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VK21
N12-CC16
N12
CC16
N12-CV13
N12
CV13
N12-DC13
N12
DC13
VK21 Peptide : VRACGADSYEMEEDGVRKCKK
CC16 Peptide : CGADSYEMEEDGVRKC
CV13 Peptide : CGADSYEMEEDGV
DC13 Peptide : DSYEMEEDGVRKC
B
Maker
N12-S1
N12-S2
EGFRvIIIex
N12-VK21
N12-CC16
N12-CV13
N12-DC13
Maker
Kd
97.4
66.2
43.0
31.0
20.1
C
Epitope Analysis
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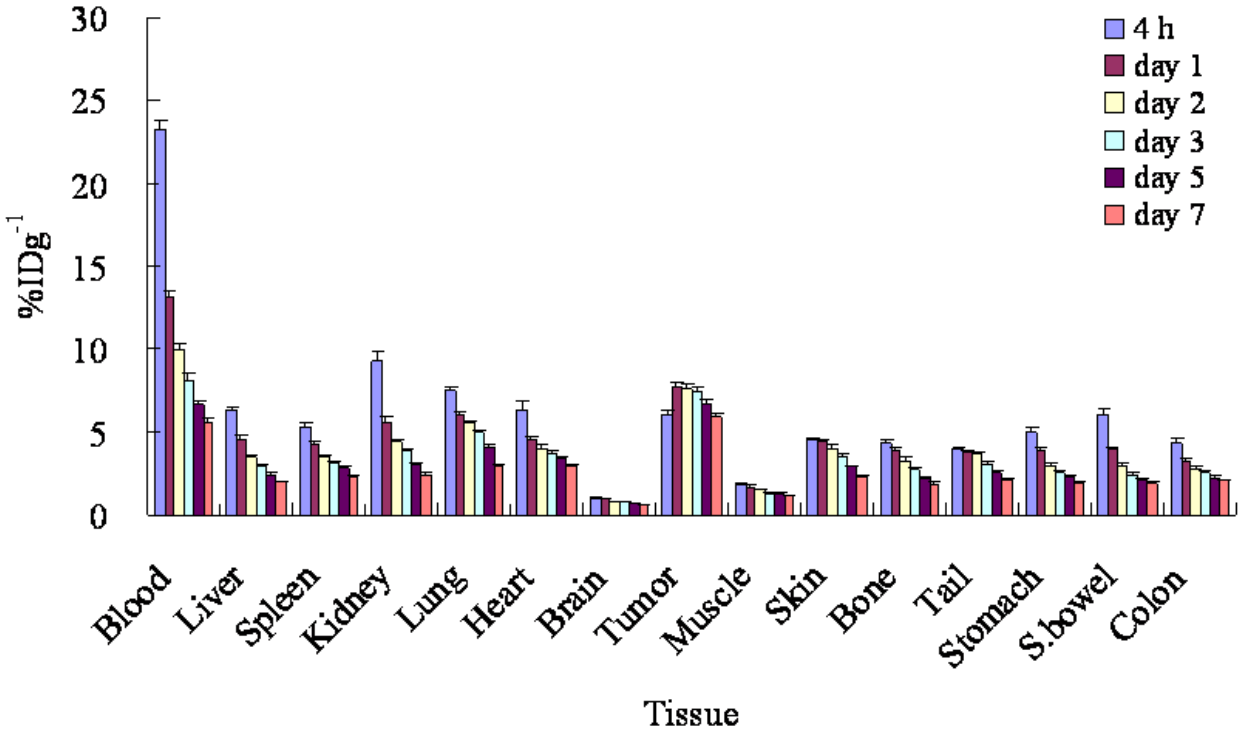
Figure S4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4 h
day 1
day 2
day 3
day 5
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Kidney
Lung
Heart
Brain
Tumor
Muscle
Skin
Bone
Tail
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S.bowel
Colon
Tissue