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Supplementary Information

Synthesis and Preclinical Evaluation of a ^{68}Ga-Labeled Peptide for PET Imaging of Transferrin Receptor 1 in Tumor

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Materials and characterization

High-performance liquid chromatography (HPLC).

Preparative HPLC was performed with a reverse-phase column (Cosmosil 5C18-AR-II, 5 μm, 20 × 150 mm, Nacalai Tesque Inc., Kyoto, Japan) connected with a guard column (Cosmosil 5C18-AR-II, 5 μm, 20 × 20 mm, Nacalai Tesque Inc.) at a flow rate of 5 mL/min; details of the solvent systems are summarized in the supplementary table (Table S1).

Analytical HPLC was performed using a reverse-phase column (Cosmosil 5C18-AR-II, 5 μm, 4.6 × 150 mm, Nacalai Tesque Inc.) at a flow rate of 1 mL/min. The detail of the solvent system is summarized in the supplementary table (Table S2).

Table S1. Systems for preparative HPLC

No.	Solvent A	Solvent B	Gradient (% of B)
1-1	0.1%TFA/H_2O	0.1%TFA/MeCN	25-75% (0-30 min)
1-2	0.1%TFA/H_2O	0.1%TFA/MeCN	25-50% (0-30 min)

Table S2. Systems for analytical HPLC

No.	Solvent A	Solvent B	Gradient (% of B)
2-1	0.1%TFA/H_2O	0.1%TFA/MeCN	25-75% (0-20 min)
2-2	0.1%TFA/H_2O	0.1%TFA/MeCN	25-50% (0-20 min)

Preparation of HBED-CC-TfRB1G3

HBED-CC-(tris(tBu))-Pr-N_3 (3): To a solution of HBED-CC-tris(tBu)ester (5.00 mg and 7.13 μmol) and 3-Azidopropylamine (1.43 mg and 14.3 μmol) in 140 μL dimethylformamide (DMF) was added WSCD·HCl (2.73 mg and 14.3 μmol) and HOBt (2.15 mg and 14.3 μmol). The reaction mixture was stirred at room temperature for 3 h. The product was analyzed by HPLC using system 2-1 (Table S2) and purified by HPLC using system 1-1 (Table S1). After removing the solvent in vacuo and lyophilization, HBED-CC-(tris(tBu))-Pr-N_3 (**3**) was obtained as a white, sticky solid (3.62 mg, 64.8%). ESI-MS: calcd for $C_{41}H_{62}N_6O_9$:783.46, found 783.50 $[M+H]^+$

HBED-CC-(tris(tBu))-Pr-TfRB1G3 (5): To a solution of HBED-CC-(tris(tBu))-Pr-N_3 (**3**) (1.48 mg and 1.46 μmol) and Hexynoyl-TfRB1G3 (**4**) (5.00 mg and 0.731 μmol) in 80% EtOH/Water (49.3 μL) was added $CuSO_4$ (5.91 mg and 3.70 μmol) and sodium ascorbate (6.52 mg and 3.70 μmol). The

reaction mixture was stirred at 60°C for 10 min. The product was analyzed by HPLC using system 2-1 (Table S2) and purified by HPLC using system 1-1 (Table S1). After purification and lyophilization, HBED-CC-(tris(tBu))-Pr-TfRB1G3 (**5**) was obtained as a white sticky solid (1.55 mg, 27.8%). ESI-MS: Calced for $C_{283}H_{442}N_{76}O_{100}S_{10}$, 1138.15157 found 1138.16581 $[M+6H]^{6+}$

HBED-CC-TfRB1G3 (6): HBED-CC-(tris(tBu))-Pr-TfRB1G3 (**5**) (1.55 mg and 0.203 μmol) was dissolved in aqueous 85 wt % phosphoric acid, and the mixture was stirred at room temperature for 5 min. The product was analyzed by HPLC using system 2-1 (Table S2) and purified by HPLC using system 1-2 (Table S1). After purification and lyophilization, HBED-CC-TfRB1G3 (**6**) was obtained as a white sticky solid (0.359 mg, 22.3%). ESI-MS: calcd for $C_{271}H_{418}N_{76}O_{100}S_{10}$, 1331.94282; found 1331.93820 $[M+5H]^{5+}$

Instant thin layer chromatography (iTLC) analysis

After radiolabeling HBED-CC-TfRB1G3 (**6**) with $[^{67}Ga]GaCl_3$ or $[^{68}Ga]GaCl_3$, 1 μL of the reaction solution was spotted on normal phase iTLC (iTLC-SG, Agilent Technologies, Santa Clara, CA) and the iTLC was developed by a mobile phase of 1 M ammonium acetate/Methanol = 1: 1 (v: v). Autoradiography (ARG) of the iTLC was obtained using phosphor imaging plates (BAS-TR 2025, Fuji Film, Tokyo, Japan) and an image analysis system (LAS7000, Multi Gauge V 3.0, Fuji Film).

Western blot analysis

U87MG cells were detached from the 24-well plate by the treatment with 250 μL Trypsin-EDTA (0.05%, Thermos Fisher Scientific Inc., Waltham, Massachusetts.). Proteins were extracted from the detached cells using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Inc., Waltham, MA) containing a protease inhibitor cocktail (cOmplete™, Roche Co., Ltd., Mannheim, Germany). The amount of the extracted proteins was determined using a BCA assay kit (PierceTM, Thermos Fisher Scientific Inc., Waltham, MA). Approximately 10 μg of total protein per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5–20% gel (e-PAGEL, ATTO Co., Ltd., Tokyo, Japan). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Q Blot Kit M, ATTO Co., Ltd., Tokyo, Japan) using a semi-dry blotting instrument. The membranes were blocked with TBST buffer (50 mM Tris–HCl, 138 mM NaCl, 2.70 mM KCl, 0.5 ml/L Tween 20, pH 8.0, Merck Sigma-Aldrich Co., St. Louis, MO) plus 5% skim milk (FUJIFILM Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for 45 min and then membranes were incubated with a human TfR1 antibody (CD71(D769X) XP Rabbit mAb, 1:4000; Cell Signaling Technology Co., Danvers, MA). Membranes were washed with TBST three times and incubated with horseradish peroxidase-labeled secondary antibody (Rabbit IgG HRP conjugate, 1:20000, Promega Co., Madison, WI). Finally, proteins were visualized and quantified using an enhanced chemiluminescence detection system (LuminoGraph I, ATTO Co., Ltd., Tokyo, Japan). Then, the membranes were incubated with anti-β-actin antibody (Anti β-actin A3854, 1:20000; Merck Sigma-Aldrich Co., St. Louis, MO), and its protein expression was analyzed using the same method as for TfR1.

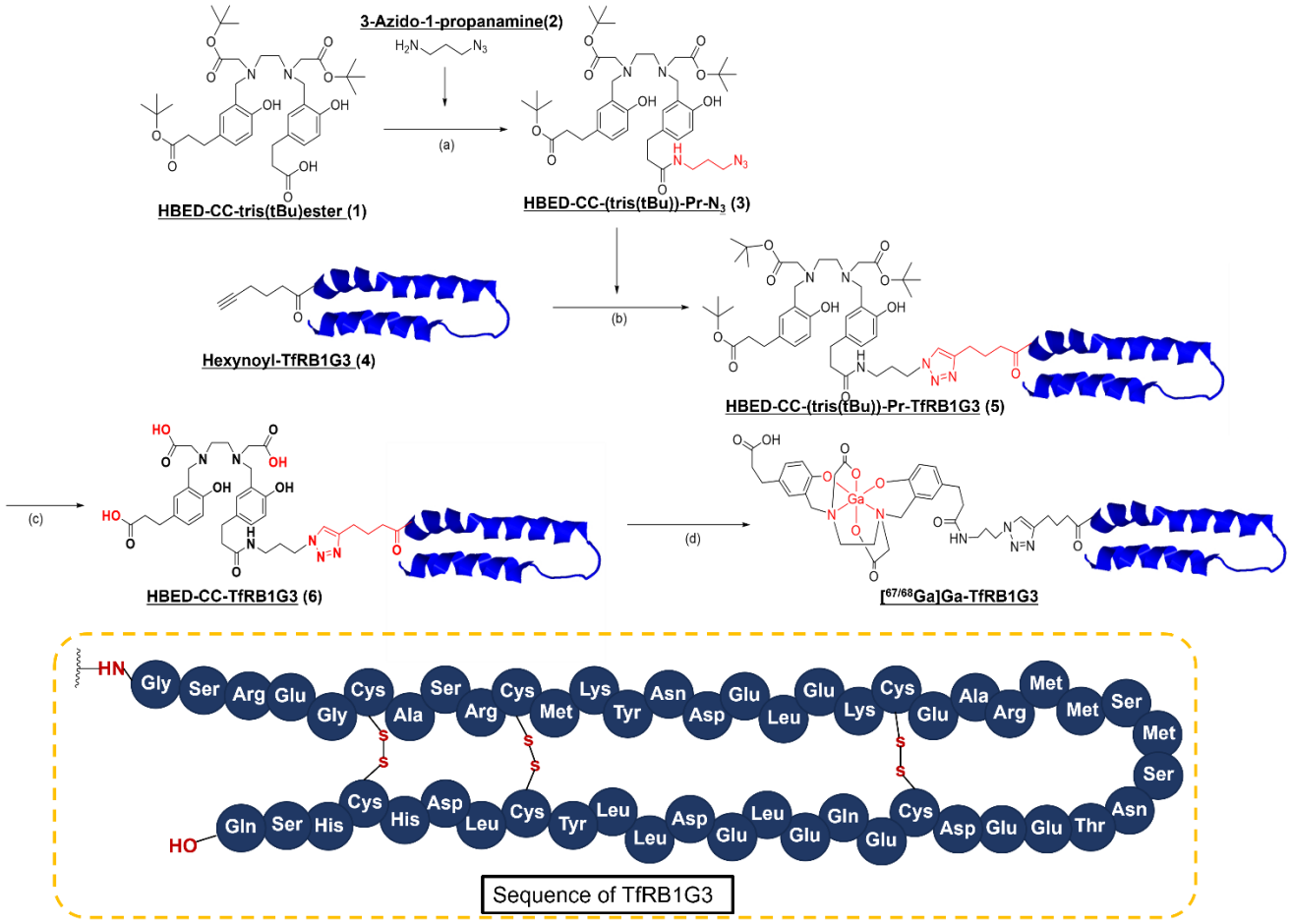

Fig. S1 Synthetic scheme for [$^{67/68}$Ga]Ga-TfRB1G3. (a) WSCD, DMF; (b) $CuSO_4$, ascorbic acid, Ethanol/Water, room temperature; (c) 85% phosphoric acid, room temperature; (d) [HBED-CC-TfRB1G3] = 5 μM, [^{67}Ga]$GaCl_3$ or [^{68}Ga]$GaCl_3$, pH 4.2, room temperature.

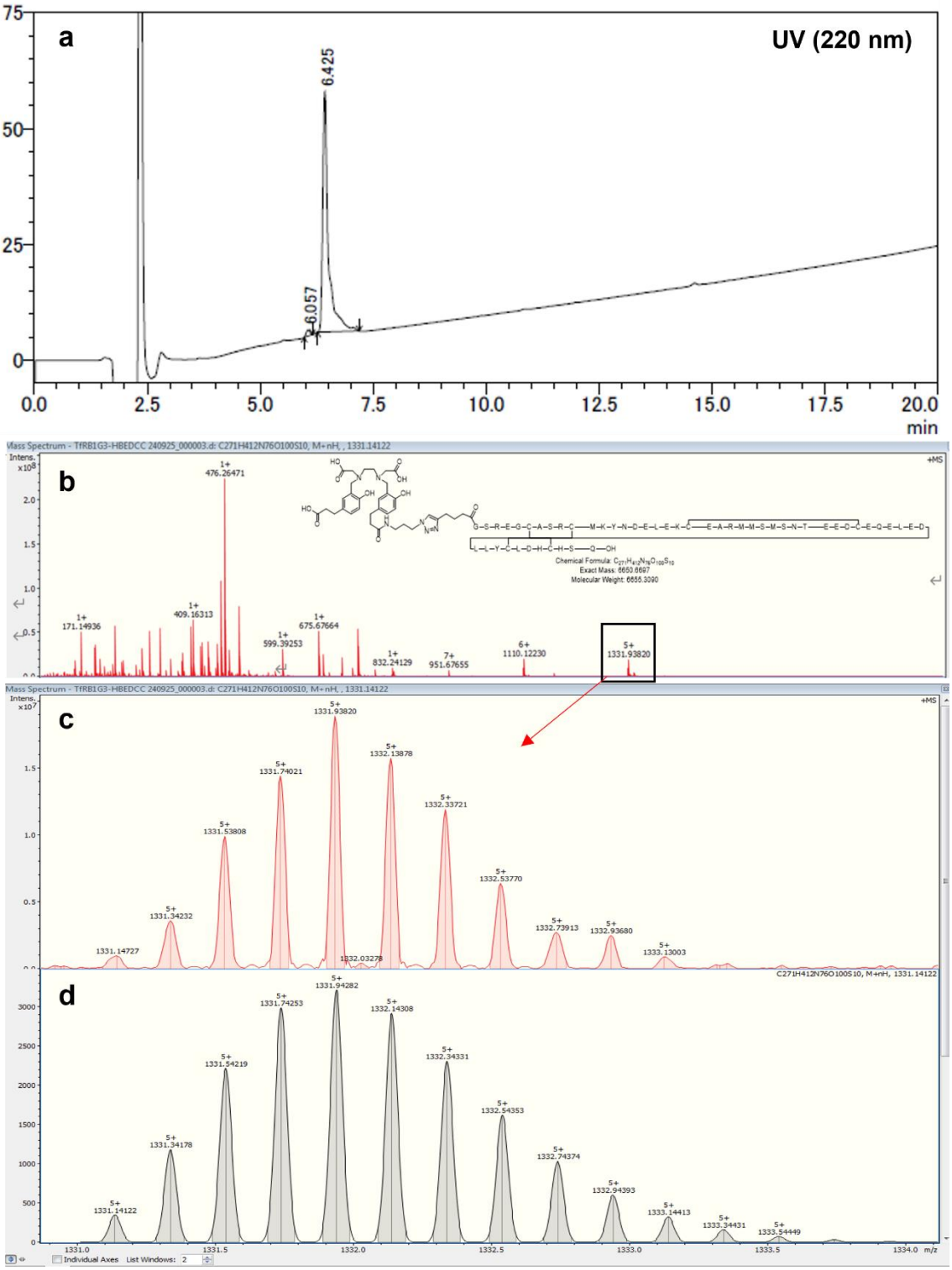

Fig. S2 (**a**) HPLC analysis of HBED-CC-TfRB1G3 (**6**) (1 nmol). (**b**) The full ESI-MS spectrum of HBED-CC-TfRB1G3 (**6**) as observed. (**c**) A magnified view of the selected spectral region. (**d**) A magnified view of the simulated ESI-MS spectrum of HBED-CC-TfRB1G3 (**6**). The observed peaks in (**c**) align precisely with the theoretical peaks calculated for $C_{271}H_{418}N_{76}O_{100}S_{10}$ (*m/z* 1331.94282, $[M + 5H]^{5+}$)

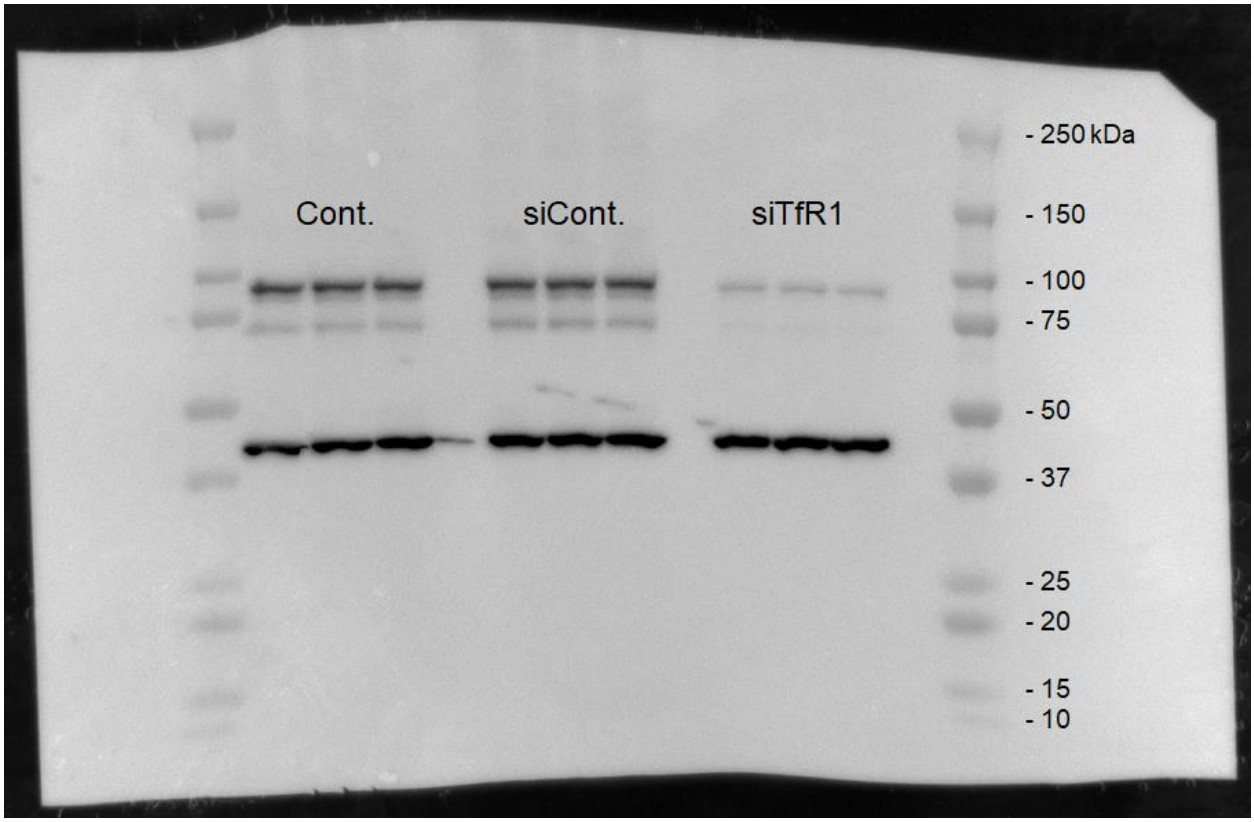

Fig. S3 The uncropped full-length blot corresponding to the western blot image shown in Figure 2a. The control group (Cont.) comprises native U87MG cells that were not treated with siRNA.

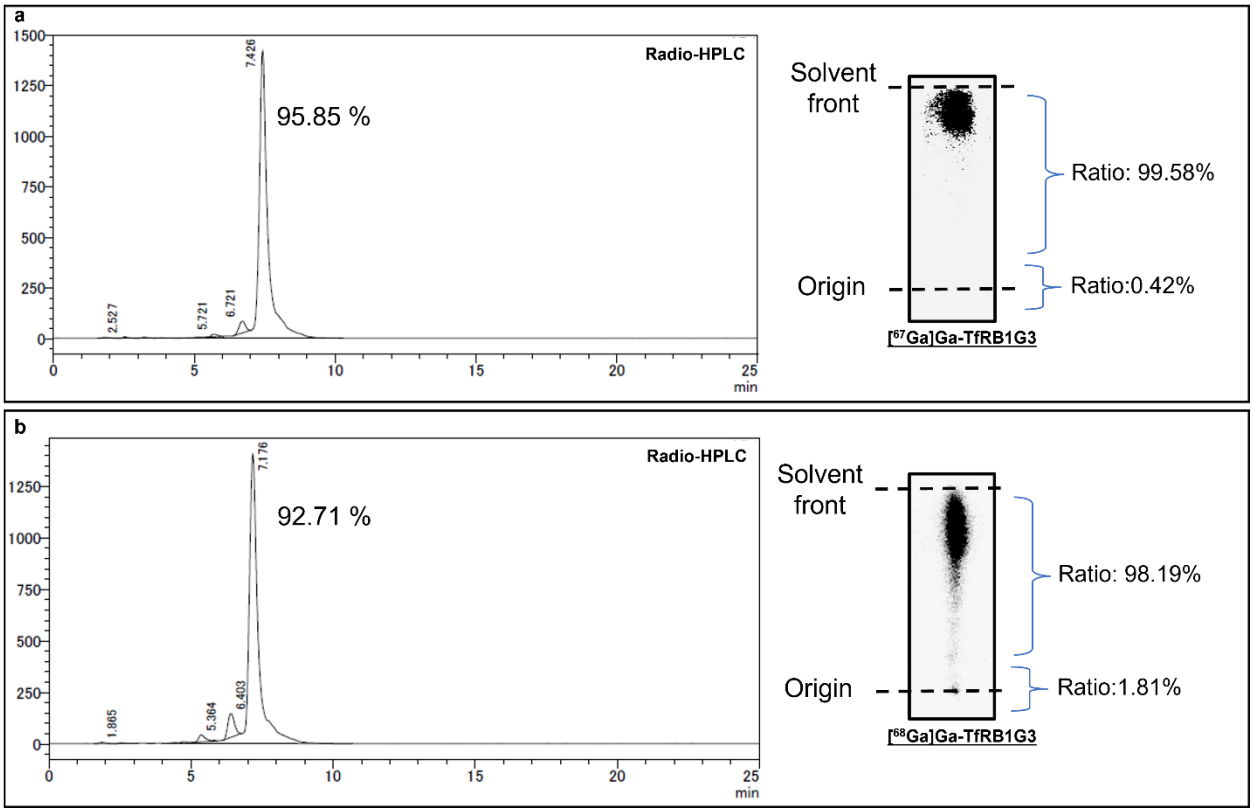

Fig. S4 (**a**) Radio-HPLC and iTLC analysis of a ^{67}Ga-labeling reaction solution of HBED-CC-TfRB1G3 (**6**). No post-labeling purification was performed. (**b**) Radio-HPLC and iTLC analysis of a ^{68}Ga-labeling reaction solution of HBED-CC-TfRB1G3 (**6**) after purification with gel filtration. Under these analytical conditions, free radiogallium elutes at a retention time of approximately 2 min in the HPLC and exhibits an Rf value of 0 in the TLC analysis.

Table S3. Result of ex vivo biodistribution study of ^{67}Ga-TfRB1G3 in U87MG tumor-bearing nude mice at 1h after injection, with and without co-injection of unlabeled TfRB1G3 as a blocking agent.

	control group		blocking group		P-value*
	Mean	SD	Mean	SD	
blood	0.45	0.054	0.38	0.044	0.118
brain	0.038	0.005	0.027	0.006	0.045
salivary glands	0.84	0.098	0.48	0.072	0.003
muscle	0.44	0.33	0.30	0.072	0.506
pancreas	1.1	0.29	0.40	0.067	0.011
heart	0.51	0.041	0.35	0.061	0.008
lung	0.88	0.077	0.72	0.106	0.063
liver	1.3	0.17	1.54	0.134	0.126
kidneys	46.3	5.46	46.3	8.06	0.987
spleen	2.2	0.39	1.47	0.155	0.035
stomach	1.5	0.19	0.68	0.05	0.0007
small intestine	3.9	0.34	1.70	0.31	0.0004
large intestine	1.4	0.24	0.42	0.041	0.0009
U87MG tumor	8.6	1.13	1.57	0.116	0.0001

*Unpaired Student's t-test.