Flow Cytometry

The αvβ6 integrin expression of HNSCC cell lines was analyzed by flow cytometry (LSR II, BD Biosciences) using a primary rat monoclonal antibody against human αvβ6 integrin (Abcam, ab97588, 3 μg/mL, 1 hour, 4°C) followed by incubation with the anti-rat Alexa Fluor 488-conjugated secondary antibody (Abcam, ab150153, 1 μg/mL, 30 min, 37°C). Antigen specificity was ensured by an isotype-matched control antibody. Each experiment was repeated at least twice, and subsequent raw data files were analyzed with FlowJo software (TreeStar).

Peptide Synthesis using Fmoc/tBU Chemistry

The peptides and their modifications were obtained by solid-phase peptide synthesis using standard Fmoc/tBu chemistry on an Applied Biosystems ABI 433A synthesizer. Removal of protecting groups and cleavage from resin was performed with 2.5% water and 2.5% triisopropylsilane in TFA for 90 min. Deprotected peptides were precipitated in cold diethyl ether. Disulfide bridge formation was performed by adding dissolved peptides (1 mg/mL) in 80% acetic acid/water to the same volume of a 0.25 mg/ml solution of iodine in acetic acid. The reaction was quenched with 20% aqueous ascorbic acid solution after 5 min. Solvents were evaporated and the residue dissolved in 50% acetonitrile in water prior to HPLC-purification (Waters XBridgeBEH130 PREP C18 column 5 μm, 19 × 150 mm). Analyses were performed on an Agilent 1100 HPLC system using a Chromolith Performance RP-C18e column 100 × 3 mm, Merck). 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B) were used as eluents. Conditions: linear gradient from 0% to 100% B within 5 min with a flow rate of 2 mL/min; UV absorbance λ = 214 nm. The identity of the peptides was verified by HPLC-MS analysis on an Agilent 1200 HPLC system equipped with a Hypersil Gold™ aq-(200 x 2.1 mm)-column (Waters) coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific).

Labeling of Peptides with 125Iodine, 68Ga or 177Lutetium

Radiolabeling with ^{125}I was conducted at the tyrosine moiety of the peptides which has been introduced in the SFTI backbone during the library construction (*12*). Labeling was performed by the chloramine-T method (Bailey, G. (2002) The chloramine T Method for Radiolabeling protein, in Protein Protocols Handbook (Walker, J., Ed) pp 963-965, Humana Press). Radiolabeled peptides were purified by analytical HPLC using a Chromolith Performance RP-18e-column, 100 × 4.6 mm (Merck). For DOTA-modifications the peptidyl resin was incubated with 2 equivalents of DOTA p-nitrophenyl-ester and 10 equivalents DIPEA in NMP for 2-16 hrs.

^{68}Ga was eluted with 0.6 M aqueous HCl from a ^{68}Ge/^{68}Ga generator (IDB Holland). 1 µL of a 1 mM solution of the respective peptide in 75 µL aqueous 2.5 M sodium acetate, 1 µL saturated ascorbic acid, and 250 ml of ^{68}Ga-generator eluate was heated at 95°C for 10 min at pH 3.6. Purification was performed with a Sola HRP 10 mg SPE cartridge (Thermo). Radiolabeled peptides were eluted with 50% aqueous ethanol. Solvents were evaporated and residual peptides dissolved in appropriate buffer.

5-25 MBq ^{177}Lu chloride (ITG Isotope Technologies, Garching) in 100 ml 0.1 M sodium acetate buffer pH 5 was added to 1 mL of a 1 mM peptide solution. The reaction mixture was heated at 90°C for 15 min and used directly for cell based experiments. The reaction mixture was diluted with 0.9% NaCl (Braun) for animal studies or purified by solid phase extraction for serum stability assays.

Serum Stability Assay

15.7 MBq of the purified ^{177}Lu-radiolabeled peptide was incubated in 200 mL human serum (Sigma-Aldrich) at 37°C. After different time intervals (10 min, 60 min 120 min, 240 min, 24 hours) 10 mL of serum was precipitated with 20 mL acetonitrile. The stability of the labeled peptide was monitored by radio-high pressure liquid chromatography of the supernatant (gradient: 0% to 40% B in 10 min; flow rate: 2 mL/mL; chromolith performance RP18ec (3 mm x

100 mm, Merck). For the detection of radioactive compounds the Agilent 1100 system was equipped with a gamma detector (Packard COBRA™ Auto-Gamma, GMI).

SUPPLEMENTAL TABLE 1: Amino acid sequences of SFITGv6 and the natural αvβ6 integrin-specific ligands grafted into the binding loop of the SFTI-1 scaffold. αvβ6 integrin-specific binding motives are in bold.

Peptide name	Binding sequence	Gene symbol	Protein name	AS length	NCBI reference sequence	m/z calculated $[M+2H]^{2+}$	m/z determined $[M+2H]^{2+}$	Retention time [min]	Peptide sequence
SFIFN1	G**RGD**SPAS	FN1	Fibronectin	2386	AAI43755.1	819.8410	819.8124	7.78	GRCTG**RGD**SPASCYPD
SFTNC	R**RGD**MSSN	TNC	Tenascin C	2201	NP_002151.2	907.8776	907.8453	5.23	GRCTR**RGD**MSSNCYPD
SFVTN	T**RGD**VFTM	VTN	Vitronectin	478	NP_000629.3	917.8896	917.8809	10.66	GRCTT**RGD**VFTMCYPD
SFLAP1	R**RGDL**AFI	TGFB1	Transforming growth factor β1	390	NP_000651.3	897.4303	897.3944	8.55	GRCTR**RGDL**AFICYPD
SFLAP3	G**RGDL**GR**L**	TGFB3	Transforming growth factor β3	412	NP_001316868.1	868.4094	868.3787	9.04	GRCTG**RGDL**GR**L**CYPD
SFITGv6	F**RGDL**MQ**L**	N/A	N/A	N/A	N/A	936.4211	936.3886	10.58	GRCTF**RGDL**MQ**L**CYPD

SUPPLEMENTAL TABLE 2: Tumor-to-Tissue Ratio of ^{177}Lu-DOTA-SFLAP3 in (A) HNO97 xenografts, (B) HNO399 xenografts, (C) HNO223 xenografts, and (D) ^{177}Lu-DOTA-SFITGv6 in HNO97 xenografts.

Supplemental Table 2A

Tumor-to-Tissue	30 min	60 min	120 min	240 min	360 min
Tumor-to-Blood	5.64±2.86	23.42±12.4	104.48±46.69	296.35±27.2	482.79±201.51
Tumor-to-Heart	14.00±9.04	44.74±9.7	92.17±17.34	124.52±21.91	118.57±32.97
Tumor-to-Lung	3.57±1.63	7.92±0.83	19.92±3.01	21.02±5.64	28.47±7.44
Tumor-to-Spleen	20.71±10.2	62.70±25.09	104.92±17.3	107.93±6.05	94.87±39.27
Tumor-to-Liver	19.30±8.89	42.84±12.74	68.88±11.38	68.12±6.45	53.82±16.65
Tumor-to-Kidney	0.56±0.26	0.94±0.05	1.02±0.2	0.80±0.15	0.76±0.2
Tumor-to-Muscle	15.72±7.72	34.83±6.16	55.90±10.06	73.70±13.73	74.81±16.16
Tumor-to-Intestine	5.16±1.7	8.77±1.6	11.38±2.91	16.42±1.0	14.07±1.94
Tumor-to-Brain	1178.69±750.21	741.00±649.95	847.17±347.64	1073.32±773.31	434.58±88.93

Supplemental Table 2B

Tumor-to-Tissue	30 min	60 min	120 min	240 min	360 min
Tumor-to-Blood	3.03±1.38	8.85±3.63	18.08±7.13	35.07±1.71	51.82±14.03
Tumor-to-Heart	5.23±2.2	10.37±5.11	14.23±4.66	21.45±3.49	19.39±4.31
Tumor-to-Lung	1.15±0.25	1.98±0.56	2.69±0.53	4.07±1.43	3.18±0.83
Tumor-to-Spleen	11.38±6.26	24.60±14.58	20.20±3.25	38.59±14.18	30.22±12.07
Tumor-to-Liver	7.78±3.05	17.07±6.04	18.27±4.97	18.41±5.5	16.39±3.38
Tumor-to-Kidney	0.24±0.12	0.33±0.12	0.24±0.1	0.24±0.07	0.23±0.04
Tumor-to-Muscle	4.85±1.4	9.14±3.21	8.90±5.21	11.66±1.9	13.02±2.81
Tumor-to-Intestine	1.30±0.31	2.54±0.62	2.58±0.62	2.77±0.24	2.46±1.16
Tumor-to-Brain	36.63±20.61	55.18±36.86	40.89±18.02	73.90±11.91	53.27±15.39

Supplemental Table 2C

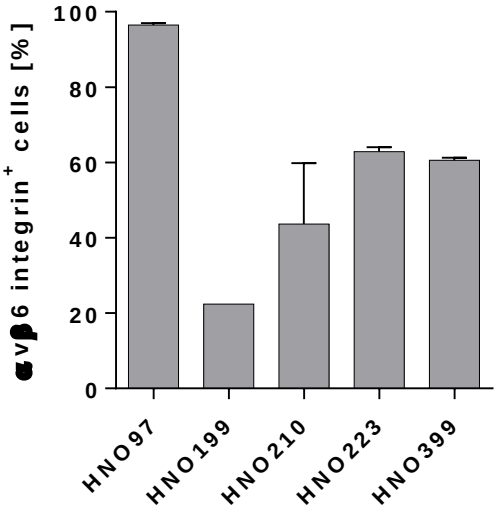
Tumor-to-Tissue	30 min	60 min	120 min	240 min	360 min
Tumor-to-Blood	2.12±1.63	8.90±2.94	17.14±2.72	57.37±6.23	86.29±58.57
Tumor-to-Heart	3.89±2.89	11.73±2.24	12.18±1.5	20.38±6.12	21.57±3.1
Tumor-to-Lung	0.67±0.5	1.47±0.34	1.48±0.22	2.80±0.45	2.45±0.52
Tumor-to-Spleen	8.08±6.35	24.97±2.93	19.61±3.94	30.68±6.15	27.27±5.81
Tumor-to-Liver	7.03±5.45	20.04±5.3	13.74±1.18	19.66±2.68	19.42±3.55
Tumor-to-Kidney	0.19±0.15	0.31±0.03	0.17±0.02	0.18±0.02	0.18±0.01
Tumor-to-Muscle	3.57±2.6	6.48±2.57	6.82±2.69	13.50±2.99	11.20±5.16
Tumor-to-Intestine	1.02±0.48	1.95±0.68	1.40±0.68	1.89±0.45	1.85±0.42
Tumor-to-Brain	22.41±17.84	63.34±9.4	27.87±23.77	64.03±20.36	58.79±16.48

Supplemental Table 2D

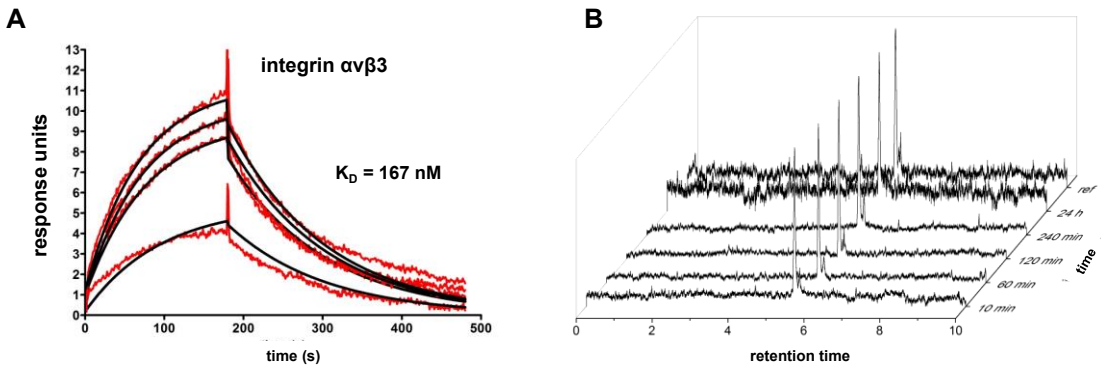
Tumor-to-Tissue	30 min	60 min	120 min	240 min	360 min
Tumor-to-Blood	6.90±0.95	11.72±1.39	41.37±6.25	55.49±15.4	72.00±19.93
Tumor-to-Heart	15.76±1.35	20.34±1.48	41.26±6.21	30.74±11.79	35.69±4.69
Tumor-to-Lung	2.65±0.31	3.37±0.81	5.31±1.57	4.11±1.67	5.85±1.68
Tumor-to-Spleen	19.30±1.54	21.24±2.27	22.93±5.17	12.67±4.36	14.75±2.44
Tumor-to-Liver	16.89±1.02	17.28±2.04	20.10±5.17	9.09±2.74	10.09±1.6
Tumor-to-Kidney	0.15±0.03	0.11±0.03	0.09±0.02	0.04±0.01	0.04±0
Tumor-to-Muscle	16.66±0.27	17.07±1.01	18.77±8.8	10.21±5.65	5.77±3.72
Tumor-to-Intestine	4.68±0.4	5.02±2.24	7.84±2.25	4.90±2.43	5.58±0.76
Tumor-to-Brain	128.71±44.46	150.60±17.54	129.21±79.45	121.31±65.5	122.58±71.73

SUPPLEMENTAL TABLE 3: Maximum Standardized Uptake Values (SUVmax) for PET/CT with ^{68}Ga-DOTA-SFLAP3 for one HNSCC patient.

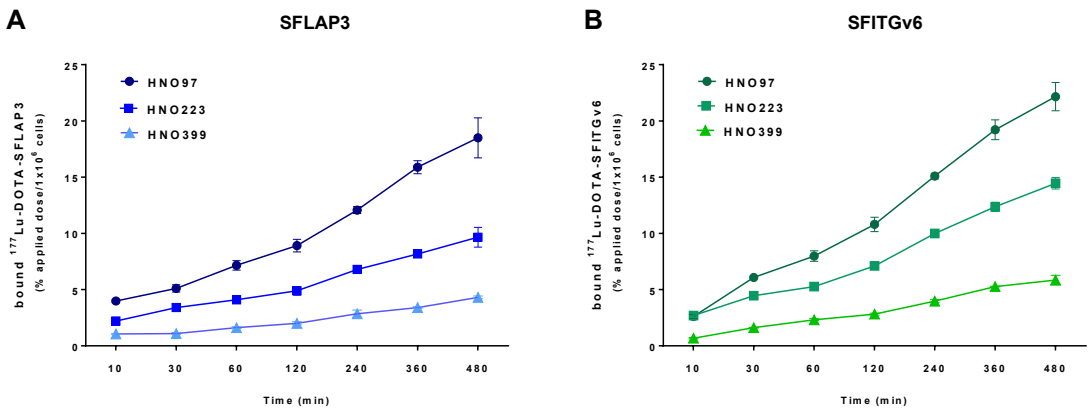
Tissue/Organ	SUVmax	
	60 min	180 min
Tumor	5.07	4.64
Hilar lymph nodes	4.07	4.15
Plexus choroideus	1.06	1.57
Cerebral parietal	0.05	0.27
Thyroid	3.27	3.24
Blood pool (mediast.)	0.99	1.05
Lung (left)	0.36	0.46
Lung (right)	0.33	0.33
Liver (left)	0.67	0.76
Liver (right)	0.73	0.75
Spleen	0.54	0.8
Pancreas head	2.3	2.62
Stomach	12.65	13.4
Duodenum	6.31	8.91
Jejunum	10.46	9.2
Ileum	4.07	17.65
Kidney (left)	60.52	36.85
Right kidney	22.43	26.41
Colon ascendens	9.2	19.37
Colon descendens	3.75	2.97
Os ileum	0.53	0.47
Muscle (glut.)	1.94	1.92



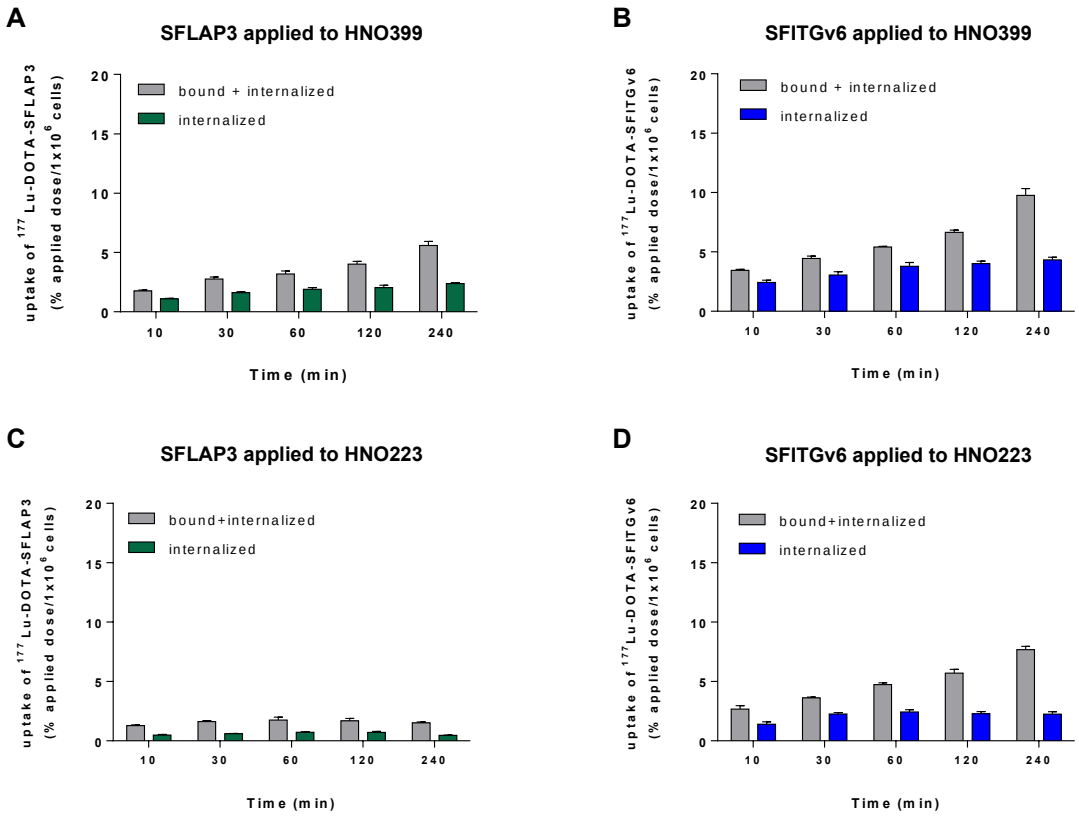
SUPPLEMENTAL FIGURE 1. Flow cytometry of αvβ6 integrin expression in the HNSCC cell lines HNO97, HNO399, HNO199, HNO210, HNO223. Bar graphs represent percentage of αvβ6 integrin-positive cells of three independent biological replicates and standard deviation.



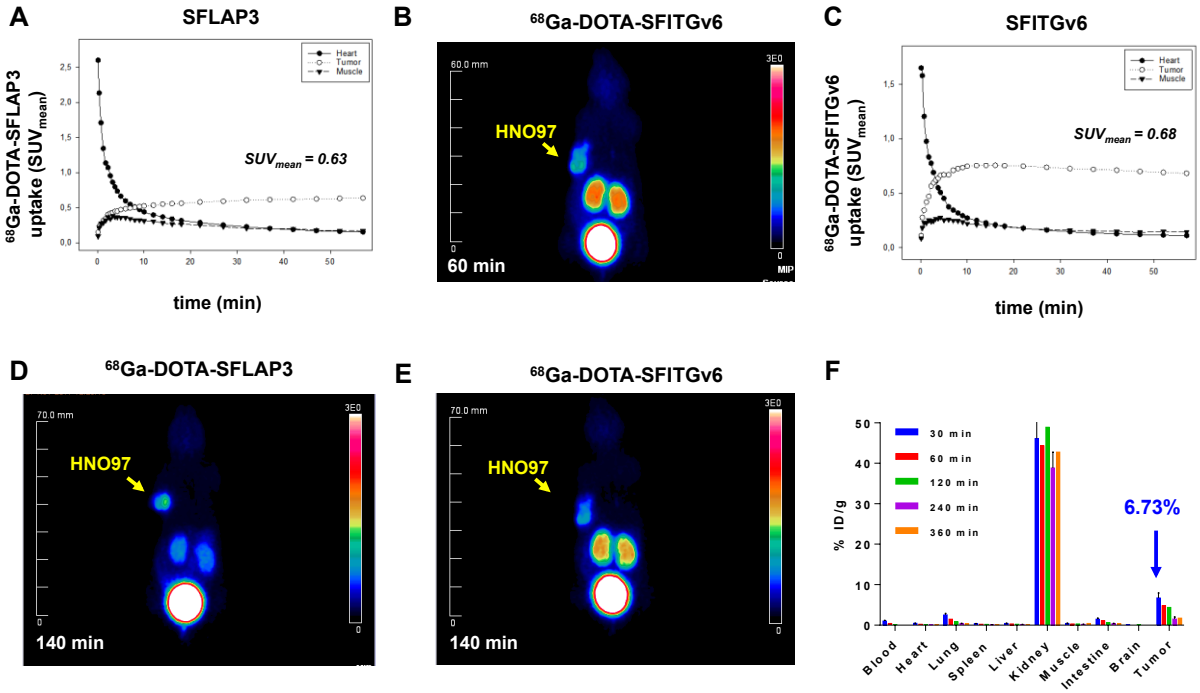
SUPPLEMENTAL FIGURE 2. (A) Affinity of αvβ3 integrin (applied concentrations: 15, 30, 35 and 40 µg/mL) for immobilized SFLAP3 (loading level: 18 response units) was measured by SPR assay (n = 3) with a flow rate of 30 mL/min. The SPR-sensograms were evaluated with the BiaCore evaluation software (black curves) and correspond to the experimentally fitted red curves. K_D values were determined by a 1:1 langmuir model fit of the SPR-sensograms. (B) The stability of ^{177}Lu-DOTA-SFLAP3 in human serum was evaluated by radio-high pressure liquid chromatography after 10, 60, 120, 240 min and 24 hrs.



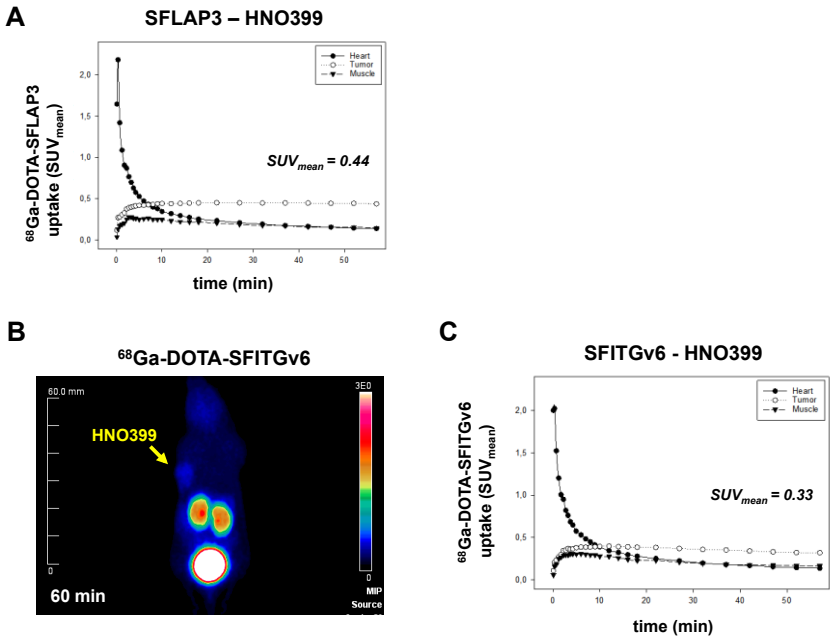
SUPPLEMENTAL FIGURE 3. Binding of ^{177}Lu-DOTA-labeled SFLAP3 (A) and SFITGv6 (B) to HNO97, HNO399, and HNO223 cells after incubation for 10, 30, 60, 120, 360, and 480 min, respectively. The amount of bound peptide (percentage of applied dose per 10^6 HNSCC cells) increased over time. Data represent mean values and standard deviation of triplicate measurements from representative experiment.



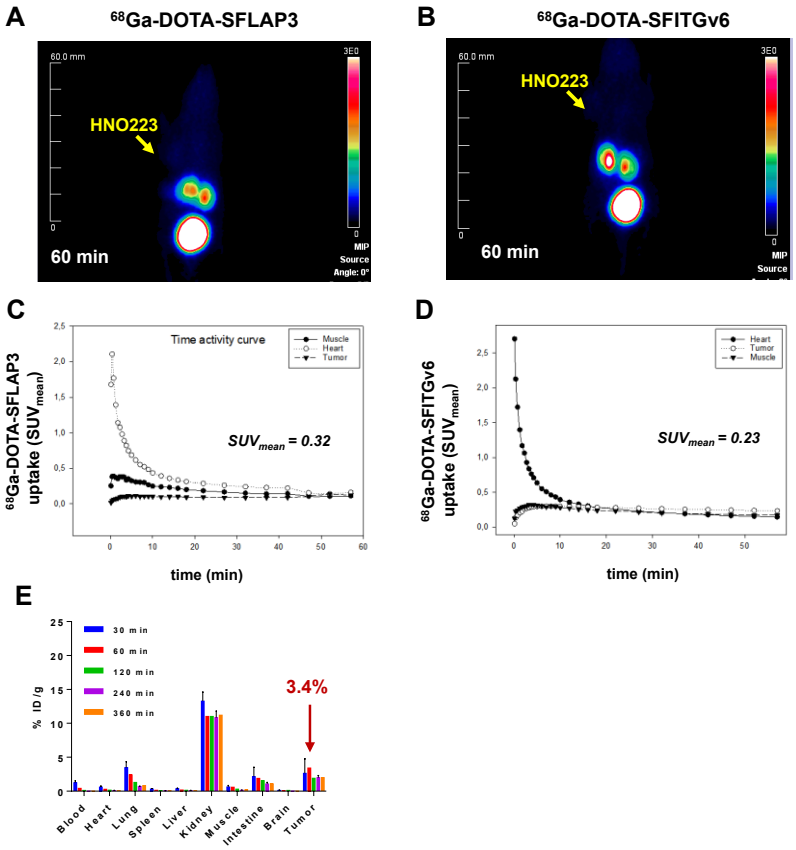
SUPPLEMENTAL FIGURE 4. Uptake (grey bars) and internalization (colored bars) of ^{177}Lu-DOTA-labeled SFLAP3 (A and C) and SFITGv6 (B and D) in HNO399 (A and B) and HNO223 cells (C and D). After incubation for 10, 30, 60, 120, and 240 min, respectively, the radioactivity in the lysates was determined and calculated as % applied dose/10^6 cells. Data represent mean values and standard deviation of triplicate measurements from representative experiment.



SUPPLEMENTAL FIGURE 5. (D) Small-animal PET imaging of HNO97 xenograft mouse 140 min after injection of ^{68}Ga-DOTA-SFLAP3 (37.9 MBq) and (A) time activity curve (SUVmean) up to 60 min. (C) Time activity curve (SUVmean) up to 60 min and small-animal PET imaging of HNO97 xenograft mouse (B) 60 min and (E) 140 min after injection of ^{68}Ga-DOTA-SFITGv6 (30 MBq). (F) Biodistribution of ^{177}Lu-DOTA-SFITGv6 (% ID/g) in tumors and organs of HNO97 xenograft mice (n=3) measured 30, 60, 120, 240, and 360 min after injection of 1 nmol (1 MBq) peptide. Data are shown as mean of three per time point and standard deviation.



SUPPLEMENTAL FIGURE 6. (A) Time activity curve (SUVmean) calculated for small-animal PET imaging of HNO399 xenograft mouse after injection of ^{68}Ga-DOTA-SFLAP3 (26 MBq). (B) Small-animal PET imaging and (C) time activity curve (SUVmean) of a HNO399 xenograft mouse 60 min after injection of ^{68}Ga-DOTA-SFITGv6 (34 MBq).



SUPPLEMENTAL FIGURE 7. (A and B) Small animal PET imaging and (C and D) time activity curves (SUVmean) of HNO223 xenograft mice 60 min after injection of (A and C) ^{68}Ga-DOTA-SFLAP3 (27 MBq) and (B and D) ^{68}Ga-DOTA-SFITGv6 (34 MBq). (E) Biodistribution of radioactivity (% ID/g) in tumors and organs of HNO223 xenograft mice (n=3) measured 30, 60, 120, 240, and 360 min after injection of 1 nmol (1 MBq) ^{177}Lu-DOTA-SFLAP3. Data are shown as mean of three per time point and standard deviation.