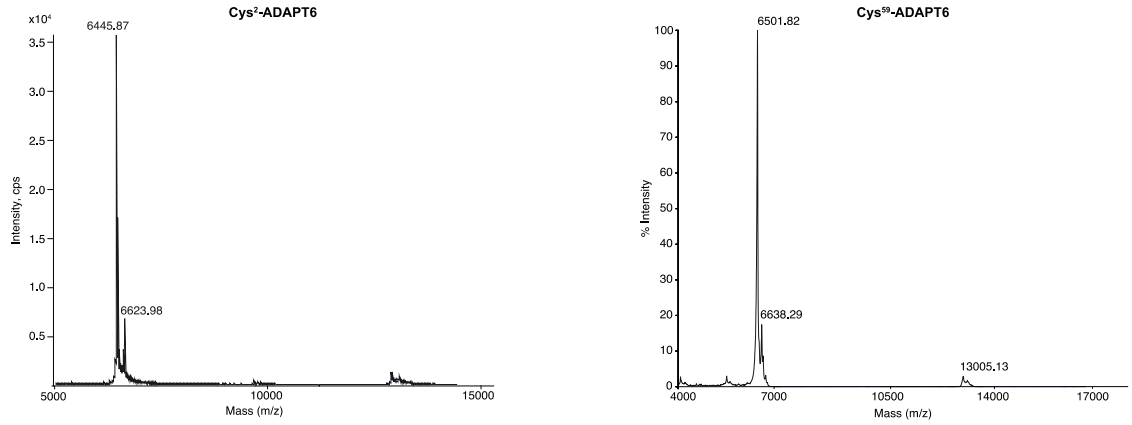
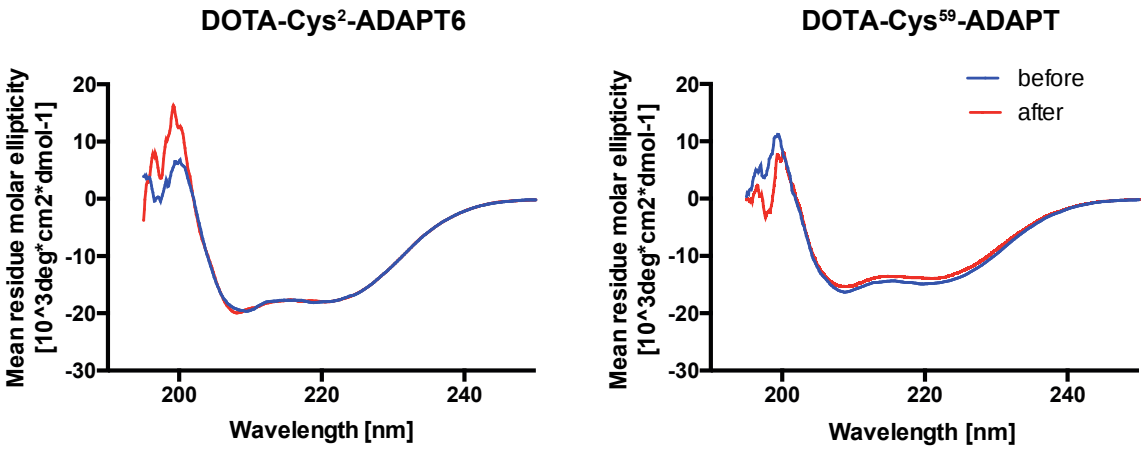
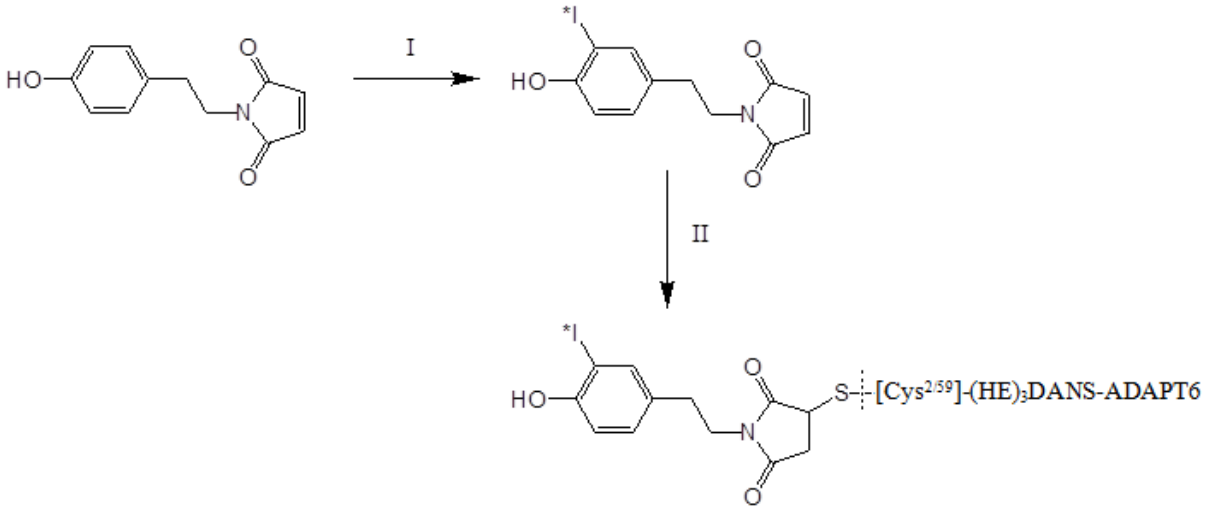


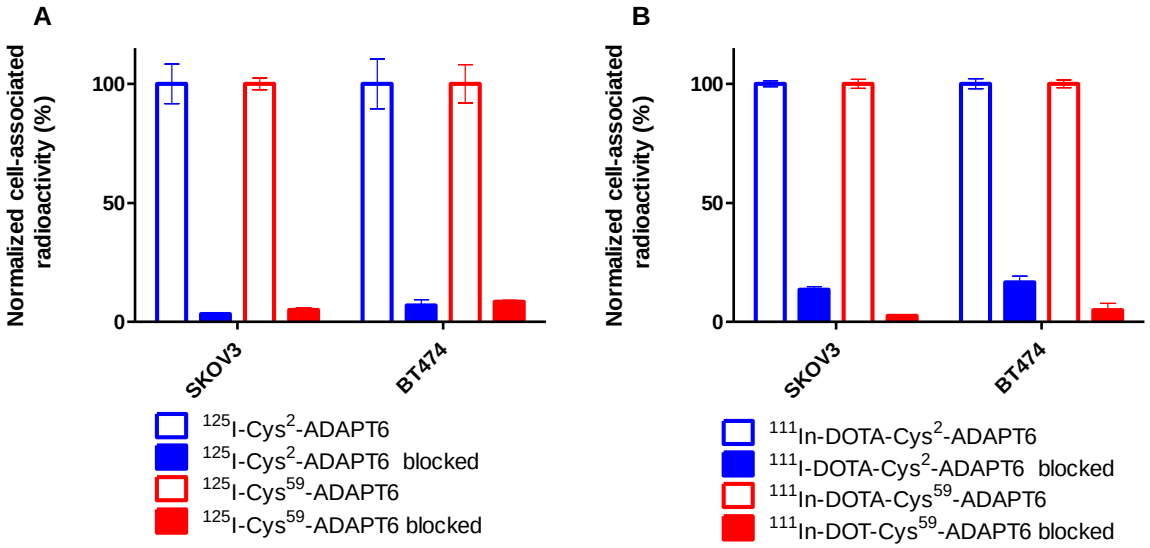
Supplemental Figure 1. Mass spectra of purified ADAPT6 variants showing the molecular weights of Cys^{2}-ADAPT6 (6447 Da) and Cys^{59}-ADAPT6 (6502 Da). Theoretical values were calculated to 6445.2 and 6502.2 Da, respectively. The molecular weight of the variants was measured by matrix assisted laser desorption ionization (MALDI) using a 4800 MALDI TOF/TOF analyzer (SCIEX).



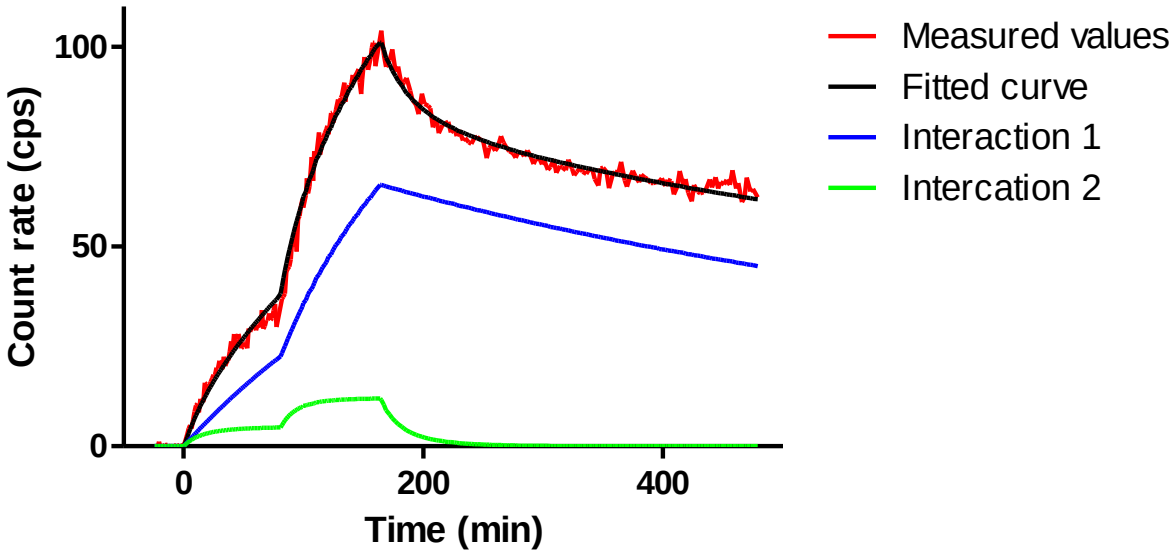
Supplemental Figure 2. Overlay of circular dichroism spectra from 250-195 nm for DOTA-Cys^{2}-ADAPT6 and DOTA-Cys^{59}-ADAPT6 before (blue) and after (red) heating from 20-90°C. The spectra show high alpha helical content of the protein domains. Also the ability to fully refold to the original secondary structure after heating to 90°C can be seen when comparing the blue and red curves. The secondary structure, melting temperature and refolding properties were determined by circular dichroism (CD) using a JASCO J-810 spectropolarimeter (JASCO).



Supplemental Figure 3. Indirect radioiodination of $Cys^{2/59}$-ADAPT6 molecules using HPEM. I: $^{125}I^-$ 5% ACOH in MeOH; Chloramine-T; 5 min at room temperature; II: freshly reduced cysteine-containing protein, ammonium acetate, pH 6.0; 60 min at room temperature



Supplemental Figure 4. Binding specificity of radiolabeled ADAPT6 constructs to HER2 expressing SKOV3 cells (1.6×10^6 receptors/cell) and BT474 cells (2×10^6 receptors/cell). (A) ^{125}I-DOTA-Cys^2-ADAPT6 and ^{125}I-DOTA-Cys^{59}-ADAPT6. (B) ^{111}In-Cys^2-ADAPT6 and ^{111}In-HPEM-Cys^{59}-ADAPT6. To test the binding specificity, 25 nM of the labeled variants was added and for receptor blocking, a 100-fold molar excess of nonlabeled corresponding ADAPT6 molecules was added. The dishes were incubated at 37°C for 1 h in a humidified incubator. The media was collected and by using trypsin-EDTA solution the cells were detached and radioactivity was measured. Data are presented as mean values with standard deviations (n=3).



Supplemental Figure 5. Representative LignadTracer sensorgram (binding of ^{111}In-DOTA-Cys^{2}-ADAPT6 to SKOV-3 cells). Uptake curves were recorded at 0.33 and 1 nM. The curves show measured data (red), a fitted curve according to the InteractionMap (black), and the two resolved interactions; one with a slower dissociation rate (blue) and one with a more rapid dissociation (green).

Supplemental Table 1. Percentage of radioactivity in the high molecular fraction (<5 kDa) of blood plasma at 1 h after injection.

	Label	
Label position	^{125}I-HPEM	^{111}In-DOTA
C-terminus	60±2	92.7±0.9
N-terminus	55±5	92.6±0.7

Supplemental Table 2. Comparison of targeting properties of some radiometal-labeled imaging probes in mice bearing SKOV-3 xenografts.

	Time point (h)	Uptake (% ID/g)				Tumor-to-organ ratio	
		Tumor	Blood	Liver	Kidney	Blood	Liver
^{111}In-DOTA-Cys^{59}-ADAPT6[a]	1	13±2	0.35±0.06	0.21±0.03	272±21	38±3	64±10
	4	15±2	0.053±0.004	0.17±0.02	284±21	277±35	85±17[c]
^{111}In-ABY-025 Affibody molecule[b]	1	17±2	1.1±0.1	1.9±0.2	186±24	15±3	8.7±0.6
	4	15±3	0.18±0.02	1.5±0.3	163±16	88±15	10±1
^{111}In-$(HE)_3$-G3 DARPin[c]	4	8.8±1.3	0.05±0.01	0.7±0.1	232±24	176	12.3
^{68}Ga-NOTA-2Rs15d nanobody[d]	1	4.2±1.0	0.5±0.2	2.9±0.3	38±6	9±3	1.4
^{111}In-CHX-A''-C6.5K-A – diabody [e]	4	7.8	5.8	5.3	27.9	1.34	1.47
	24	9.8	0.5	3.6	31	19.6	2.7
^{111}In-DTPA-trastuzumab [f]	48	16.3±0.6	7.2±0.7	10±1	6.5±0.2	2.3	1.63

[a] data from this study;

[b] data from Ahlgren S, Orlova A, Wållberg H et al. Targeting of HER2-expressing tumors using ^{111}In-ABY-025, a second-generation affibody molecule with a fundamentally reengineered scaffold. *J Nucl Med.* 2010;51:1131-1138.

[c] data from Goldstein R, Sosabowski J, Livanos M et al. Development of the designed ankyrin repeat protein (DARPin) G3 for HER2 molecular imaging. *Eur J Nucl Med Mol Imaging.* 2015;42:288-301.

[d] data from Xavier C, Vaneycken I, D'huyvetter M et al. Synthesis, preclinical validation, dosimetry, and toxicity of ^{68}Ga-NOTA-anti-HER2 Nanobodies for iPET imaging of HER2 receptor expression in cancer. *J Nucl Med.* 2013;54:776-784.

[e] data from Adams GP, Shaller CC, Dadachova E et al. A single treatment of yttrium-90-labeled CHX-A"-C6.5 diabody inhibits the growth of established human tumor xenografts in immunodeficient mice. *Cancer Res*. 2004;64:6200-6206.

[f] data form Lub-de Hooge MN, Kosterink JG, Perik PJet al. Preclinical characterisation of ^{111}In-DTPA-trastuzumab. *Br J Pharmacol* 2004;143 99-106.