Supporting Information

Renal Handling of ^{99m}Tc-Labeled Antibody Fab Fragments with a Linkage Cleavable by Enzymes on Brush Border Membrane.

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List of Supplementary Materials

GFK-Fab or [^{111}In]In-DTPA-CHX-A”-Fab in normal mice.

General. Technetium-99m (^{99m}Tc) as [^{99m}Tc]$NaTcO_4$ was eluted in saline solution daily from a ^{99}Mo/^{99m}Tc generator (Ultra-Techne Kow, FUJIFILM Toyama Chemical Co. Ltd., Tokyo). Indium-111 (^{111}In) chloride was purchased from Nihon Medi-Physics Co., Ltd (Tokyo). Mass spectrometry was carried out using AccuTOF LC-plus (JMS-T100LP, JEOL Ltd., Tokyo) or Agilent 6130 series Quadrupole LC/MS electrospray system (Agilent Technologies, Tokyo). ^{1}H-NMR spectra were recorded on a JEOL JNM-ECS-400 spectrometer (JEOL Ltd., Tokyo). Fmoc-Lys-OtBu, 2-((3-*tert*-bu)benzoyl)thio)acetic acid (compound 5), and tetra(*n*-butyl)ammonium-[tetrachlorooxorhenium(V)] (TBA[$ReOCl_4$] were synthesized according to the procedures reported previously.(*14, 28, 29*) The BBMVs were isolated from the renal cortex of male Wistar rats by the Mg/EDTA precipitation method, as described previously.(*30*) Cl-Trt(2-Cl)-resin was obtained from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). [(R)-2-Amino-3-(4-isothiocyanatophenyl)propyl]-trans-(S,S)- cyclohexane-1,2-diaminepenta-acetic acid (*p*-SCN-Bn-CHX-A"-DTPA) was purchased from Macrocyclics Inc. (Plano, U.S.A.) A monoclonal antibody against c-kit (12A8) was obtained from Immuno-Biological Laboratories (Takasaki, Japan). The Fab fragment of 12A8 was prepared using a Fab preparation kit (Pierce, Rockford, IL), as reported previously.(*10*) All commercially available chemicals were of analytical or reagent grade and were used without further purification. All compounds tested in in vitro and in vivo studies were prepared in >95% purities as determined by HPLC.

1. Synthetic procedures for MAG_3-GFK-suc-BA and MAG_3-GFK-suc-TFP.

Fmoc-Lys(suc)-OtBu (3). A solution of Fmoc-Lys-OtBu (400 mg, 1.32 mmol) in acetonitrile (5 mL) was added succinic anhydride (140 mg, 1.32 mmol). After mixing for 4 h, the solvent was evaporated *in vacuo,* and the residue was purified with silica gel column chromatography ($CHCl_3$/MeOH = 10/1) to afford compound 1 as a colorless oil (292 mg, 42%). ^{1}H-NMR ($CHCl_3$): δ 1.45 (15H, overlapped, tBu, CH_2), 2.37 (2H, t, CH_2CO), 2.66 (2H, m, CH_2COOH), 3.24 (2H, m, CH_2NH), 4.16 (2H, d, $CHCH_2O$), 4.34 (2H, overlapped, CH(Fmoc), CH(Lys)), 5.78 (2H, m, NH, NH), 7.44 (10H, m, Fmoc). ESI-MS $(M+Na)^+$: m/z 547, found: 547.

18-Benzyl-21-(*tert*-butoxycarbonyl)-1-(3-(*tert*-butoxycarbonyl)phenyl)-1,4,7,10,13,16,19,27-octaoxo-2-thia-5,8,11,14,17,20,26-heptaazatriacontan-30-oic acid (6). The intermediate compound, H-$(Gly)_4$-Phe-Lys(suc)-OtBu (4), was synthesized by the Fmoc solid-phase synthesis using Cl-Trt(2-Cl) resin. Fmoc-Lys(suc)-OtBu, Fmoc-Phe-OH, and Fmoc-Gly-OH were used as the protecting amino acids. The peptide chain was constructed manually according to the published method consisting of (I) 20 min of deprotection with 20% piperidine-dimethylformamide (DMF) and (II) 2 h of coupling of amino acid derivatives (2.8 equiv.) with *N*,*N*-diisopropylcarbodiimide (DIC, 2.8 equiv.) and 1-hydroxybenzotriazole (HOBt, 2.8 equiv.) in DMF (3 mL). After

assembling the respective amino acid, the peptide resin was reacted with 2-[[3-(tert-butoxycarbonyl)benzoyl]thio]acetic acid in the presence of HOBt and DIC for 2 h. The peptide resin was then treated with a mixture of acetic acid/2,2,2-trifluoroethanol/dichloromethane (3/1/6; 5 mL) for 2 h in to remove the peptide from the resin. After removing the resin by filtration, the filtrate was treated with diethyl ether to precipitate 6 as a white solid (121 mg, 87.5%). ^{1}H-NMR (DMSO) : δ 1.31-1.64 (15H, overlapped, CH_2CH_2), 1.39 (9H, s, *tert*-butyl), 1.57 (9H, s, *tert*-butyl), 2.26-2.29 (2H, t, $COCH_2CH_2$), 2.37-2.41 (2H, t, $COCH_2CH_2$), 3.01-3.04 (4H, overlapped, $NHCH_2CH_2$, $CHCH_2C_6H_5$), 3.71-3.80 (8H, overlapped, $NHCH_2CO$), 3.92 (2H, s, SCH_2CO), 4.05 (1H, q, NHCHCO), 4.53-4.58 (1H, q, NHCHCO), 7.17-7.25 (5H, m, *aromatic*), 7.70-7.73 (1H, m, *aromatic*), 7.88-8.57 (10H, overlapped, $CONHCH_2$, CONHCH, *aromatic*). ESI-MS $(M+Na)^+$: *m/z* 978, found: 978. ESI-MS $(M+Na)^+$: m/z 978, found: 978.

***tert*-Butyl 3-(18-benzyl-21-(*tert*-butoxycarbonyl)-4,7,10,13,16,19,27,30-octaoxo-30-(2,3,5,6-tetrafluorophenoxy)-2-thia-5,8,11,14,17,20,26-heptaazatriacontanoyl)benzoate (7).** To a chilled solution of 4 (33 mg, 0.04 mmol) and 2,3,5,6-tetrafluorophenol (14.3 mg, 0.09 mmol) in DMF (100 mL) was added dropwise a solution of DIC (13.0 mL, 0.09 mmol) in DMF (50 mL) to maintain the temperature (0 ºC), and the reaction mixture was stirred at room temperature overnight. Diethyl ether (5 mL) was added to the solution to precipitate compound 6 as a white solid (20 mg, 52.5%). ^{1}H-NMR (DMSO): δ 1.39 (9H, s, *tert*-butyl), 1.48 (6H, m, CH_2CH_2), 1.57 (9H, s, *tert*-butyl), 2.99 (8H, overlapped, $COCH_2CH_2$, $NHCH_2CH_2$, $CHCH_2C_6H_5$), 3.76 (8H, overlapped, $NHCH_2CO$), 3.92 (2H, s, SCH_2CO), 4.05 (1H, q, NHCHCO), 4.56 (1H, q, NHCHCO), 7.21 (5H, m, *aromatic*), 7.72 (1H, m, *aromatic*), 8.23 (11H, overlapped, $CONHCH_2$, CONHCH, *aromatic*). ESI-MS $(M+H)^+$: *m/z* 1104, found: 1104.

18-Benzyl-1-(3-carboxyphenyl)-1,4,7,10,13,16,19-heptaoxo-21-(4-(4-oxo-4-(2,3,5,6-tetrafluorophenoxy)butanamido)butyl)-2-thia-5,8,11,14,17,20-hexaazadocosan-22-oic acid (MAG_3-GFK-suc-TFP) (1). Compound 5 (20 mg, 0.02 mmol) was dissolved in 10% anisole/TFA (1 mL) and was kept at room temperature for 2 h. Diethyl acetate (10 mL) was then added to the solution to precipitate the white solid. The white solid was washed by diethyl ether (30 mL) to afford compound 1 as a white crystal (14.5 mg, 80.6%). ^{1}H-NMR (DMSO): δ1.39 (9H, s, *tert*-butyl), 1.48 (6H, m, CH_2CH_2), 2.99 (8H, overlapped, $COCH_2CH_2$, $NHCH_2CH_2$, $CHCH_2C_6H_5$), 3.76 (8H, overlapped, $NHCH_2CO$), 3.92 (2H, s, SCH_2CO), 4.05 (1H, q, NHCHCO), 4.56 (1H, q, NHCHCO), 7.21 (5H, m, *aromatic*), 7.72 (1H, m, *aromatic*), 8.23 (11H, overlapped, $CONHCH_2$, CONHCH, *aromatic*). ESI-MS $(M+H)^+$: *m/z* 992, found: 992.

2-(Tritylthio)acetic acid (8). Thioglycolic acid (0.40 mL, 5.73 mmol) and triphenylmethanol (1.49 g, 5.73 mmol) were dissolved in TFA (18 mL), and the mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure, and toluene (5 mL) was added to the residue for azeotropic. This process was

repeated for three times. After removing toluene, hexane was added to the pale orange oily residue, and the left in the refrigerator overnight. The white precipitate was collected, washed with hexane to provide 8 as a white crystal (1.81 g, 94.3%). ^{1}H NMR (DMSO): δ 2.83 (2H, s, SCH_2CO), 7.24-7.37 (15H, m, *aromatic*). ESI-MS $(M+Na)^+$: *m/z* 357, found: 357.

18-Benzyl-21-(*tert*-butoxycarbonyl)-4,7,10,13,16,19,27-heptaoxo-1,1,1-triphenyl-2-thia-5,8,11,14,17,20,26-heptaazatriacontan-30-oic acid (9). Compound 4 (0.19 mmol) was reacted with 8 (79.6 mg, 0.24 mmol) in the presence of HOBt (36.4 mg, 0.24 mmol) and DIC (36.6 μL, 0.24 mmol) in DMF (1 mL) at room temperature overnight. After confirming the absence of a free amine group by Kaiser test, the resin was treated with a mixture of acetic acid/2,2,2-trifluoroethanol/dichloromethane (3/1/6; 5 mL) for 2 h in to remove the peptide from the resin. After removing the resin by filtration, the filtrate was treated with diethyl ether to precipitate 9 as white solids (79.6 mg, 84.3%). ^{1}H-NMR (DMSO) : δ 1.30-1.50 (4H, m, $CH_2CH_2CH_2$), 1.39 (9H, s, *tert*-butyl), 1.55~1.68 (2H, m, $CHCH_2CH_2$), 2.26-2.29 (2H, t, $COCH_2CH_2$), 2.37-2.41 (2H, t, $COCH_2CH_2$), 2.85 (2H, s, SCH_2CO), 3.01-3.04 (4H, overlapped, $NHCH_2CH_2$, $CHCH_2C_6H_5$), 3.41-3.72 (8H, overlapped, $NHCH_2CO$), 4.02-4.08 (1H, q, NHCHCO), 4.53-4.58 (1H, q, NHCHCO), 7.17-7.27 (5H, m, *aromatic*), 7.31-7.36 (15H, m, *aromatic*), 7.88-8.34 (7H, overlapped, $CONHCH_2$, CONHCH). ESI-MS $(M+Na)^+$: *m/z* 1016, found: 1016.

***tert*-Butyl N^6-(4-(benzylamino)-4-oxobutanoyl)-N^2-(2-(tritylthio)acetyl)glycylglycyl-glycylglycylphenylalanyllysinate (10).** To a mixed solution of 9 (40 mg, 0.04 mmol), benzylamine (4.8 μL, 0.04 mmol), and N-hydroxysuccinimide (5.06 mg, 0.04 mmol) in DMF was added a DMF solution of DIC (13.5 mL, 0.09 mmol) dropwise at 0 °C. The reaction mixture was kept overnight at room temperature, and the solvent was removed in vacuo. The residue was dissolved in ethanol (3 mL) and treated with ether (12 mL) to precipitate the crude product. The RP-HPLC purification of the crude product provided compound 10 in 38.8% (16.8 mg) yield. ^{1}H-NMR (CD_3OD): δ 1.42 (4H, m, $CH_2CH_2CH_2$), 1.45 (9H, s, tert-butyl), 1.73 (2H, m, $CHCH_2CH_2$), 2.51 (4H, s, $COCH_2CH_2$), 2.95 (2H, q, $CHCH_2C_6H_5$), 307 (2H, s, SCH_2CO), 3.16 (2H, $CHCH_2C_6H_5$), 3.77 (8H, overlapped, $NHCH_2CO$), 4.23 (1H, q, NHCHCO), 4.35 (2H, s, $NHCH_2C_6H_5$), 4.63 (1H, q, NHCHCO), 7.30 (25H, overlapped, aromatic). ESI-MS $(M+Na)^+$: 1105, found: 1105.

Trt-MAG_3-Gly. Trt-MAG_3-Gly was synthesized according to the procedure mentioned above using H-$(Gly)_4$-Trt(2-Cl) and compound 8. After removed by filtration, the filtrate was treated with ether to provide Trt-MAG3-Gly as a white solid in 62.2% yield. ^{1}H-NMR (DMSO): δ 2.86 (2H, s, SCH_2CO), 3.69 (8H, overlapped, $NHCH_2CO$), 7.31 (15H, m, aromatic), 8.16 (3H, overlapped, $CONHCH_2$). ESI-MS $(M+Na)^+$: m/z 585, found: 585.

Trt-MAG$_3$. This compound was synthesized according to the procedure mentioned above using H-(Gly)$_3$-Trt(2-Cl) in 84.1% yield. ^{1}H-NMR (DMSO): δ 2.85 (2H, s, SCH_2CO), 3.69 (6H, overlapped, $NHCH_2CO$), 7.31 (15H, m, aromatic), 8.16 (3H, overlapped, $CONHCH_2$). ESI-MS $(M+Na)^+$: m/z 528, found: 528.

2. Synthetic procedures for non-radioactive $^{185/187}$Re complexes of MAG$_3$, MAG$_3$-Gly, and MAG$_3$-GFK-suc-BA.

Trt-MAG$_3$, Trt-MAG$_3$-Gly, or 10 (1.60 μmol each) was treated with a mixture of triethylsilane (10 μL) and TFA (190 μL) at room temperature for 2 h. After removing the solvent under a stream of N_2, ether was added to the residue to precipitate the deprotected ligand. The ligand was washed with ether to remove the organic solvent several times and dissolved in water (50 μL) containing sodium acetate (1 mg). To the solution was added a mixture of $(Bu_4N)[ReOCl_4]$ (0.60 mg, 1.02 μmol) and ethyleneglycol (10 μL) in ethanol (50 μL), and the reaction mixture was heated at 80 ˚C for 1 h. The RP-HPLC purification provided the respective Re complex. Re-MAG$_3$, ESI-MS $(M-)^-$: *m/z* 461, found: 461. Re-MAG$_3$-Gly, ESI-MS $(M-H)^-$: *m/z* 518, found: 518. Re-MAG$_3$-FK(suc-BA), ESI-MS $(M-)^-$: *m/z* 926, found: 926. Re-MAG$_3$-GFK-suc-BA, ESI-MS $(M+H)^+$: *m/z* 984, found: 984.

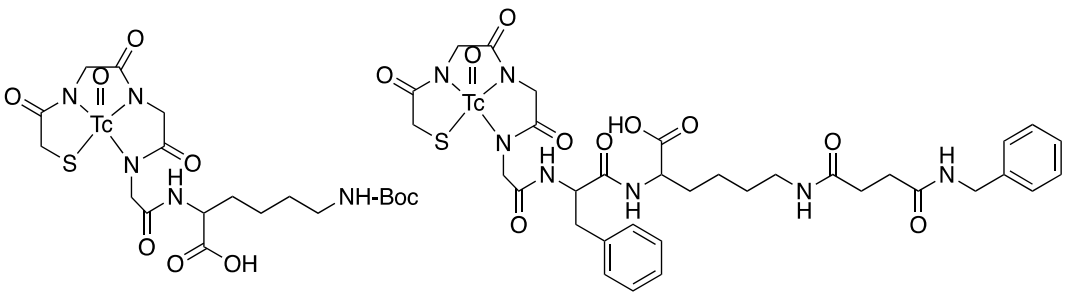


Figure S1. Chemical structures of [^{99m}Tc]Tc-MAG$_2$-GK-Boc (left) and [^{99m}Tc]Tc-MAG$_2$-GFK-suc-BA (right). The GK and the GFK linkages of [^{99m}Tc]Tc-MAG$_2$-GK-Boc and [^{99m}Tc]Tc-MAG$_2$-GFK-suc-BA failed to be recognized and cleaved by enzymes on the renal brush border membranes vesicles (BBMVs).

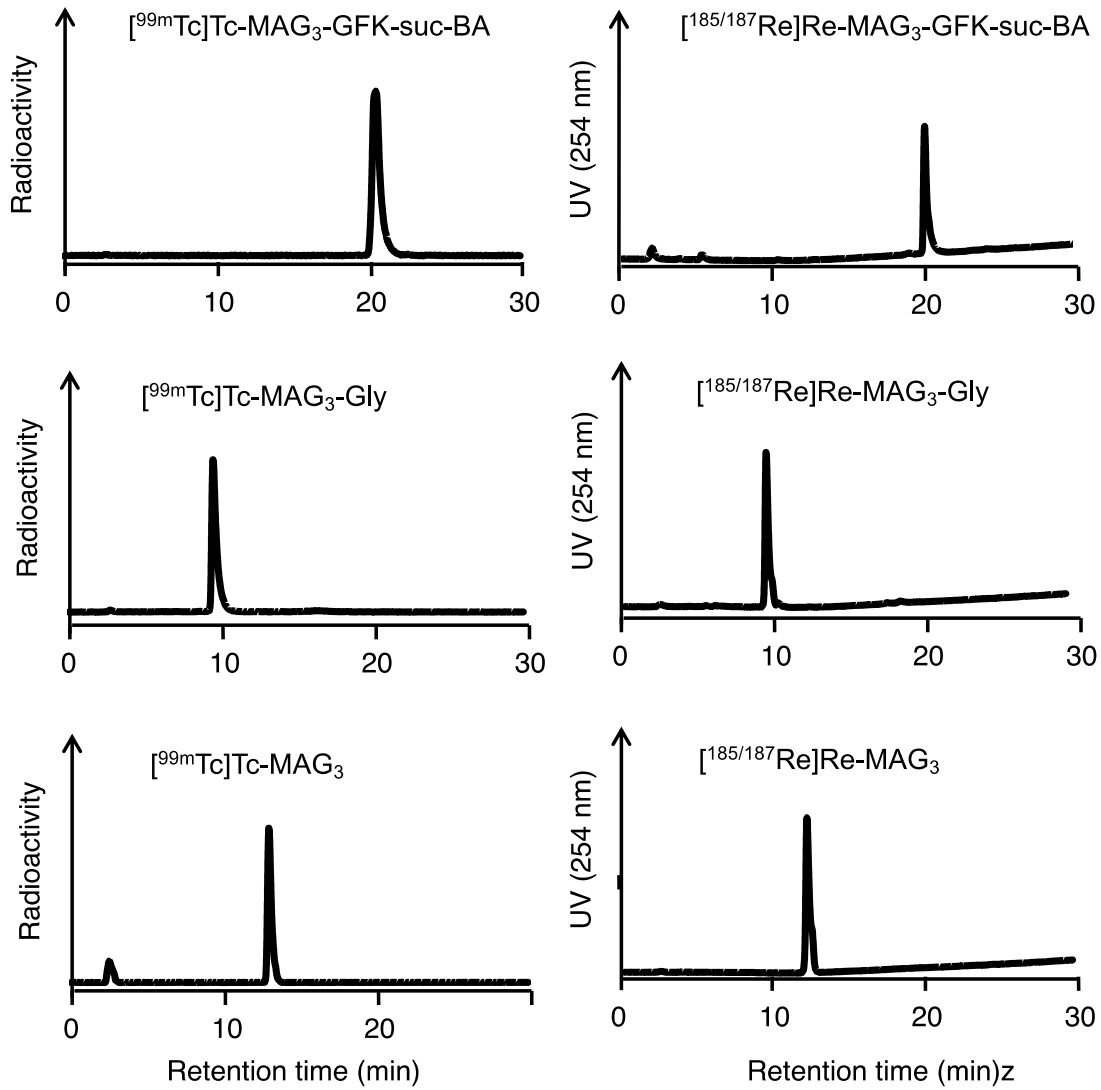


Figure S2. RP-HPLC elution profiles of [^{99m}Tc]Tc-MAG$_3$-GK-suc-BA, [^{99m}Tc]Tc-MAG$_3$-Gly, [^{99m}Tc]Tc-MAG$_3$, and their non-radioactive Re-counterparts. Radioactivity trace of each [^{99m}Tc]Tc-MAG$_3$-derivative showed a single peak at a retention times similar to those of the respectiver non-radioactive Re-counterpart.

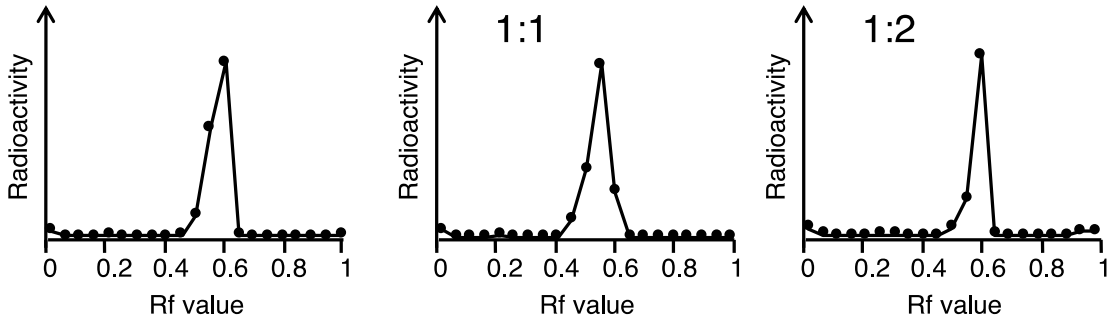


Figure S3. TLC profiles of [^{99m}Tc]Tc-MAG$_3$-GFK-suc-BA in the absence (left), and the presence of an equimolar (middle) or a twice higher molar amount (right) of Fab. Under the conditions, [^{99m}Tc]Tc-MAG$_3$-GFK-suc-BA has an Rf value of 0.55-0.6, while the protein-bound ^{99m}Tc remains at the origin.

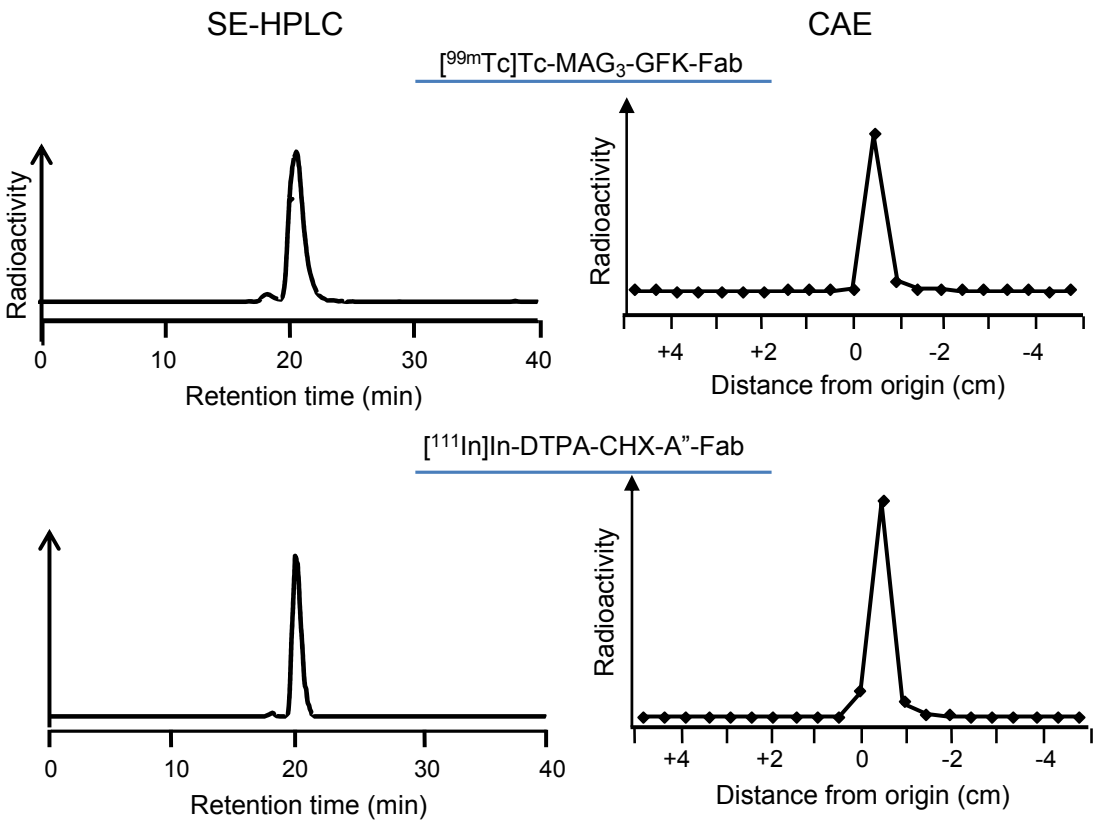


Figure S4. SE-HPLC (left) and cellulose acetate electrophoresis (right) profiles of [^{99m}Tc]Tc-MAG$_3$-GFK-Fab (top) and [^{111}In]In-DTPA-CHX-A"-Fab (bottom).

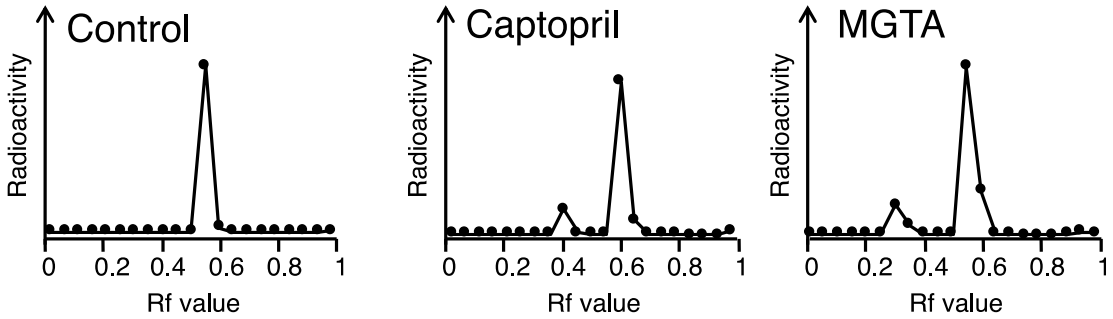
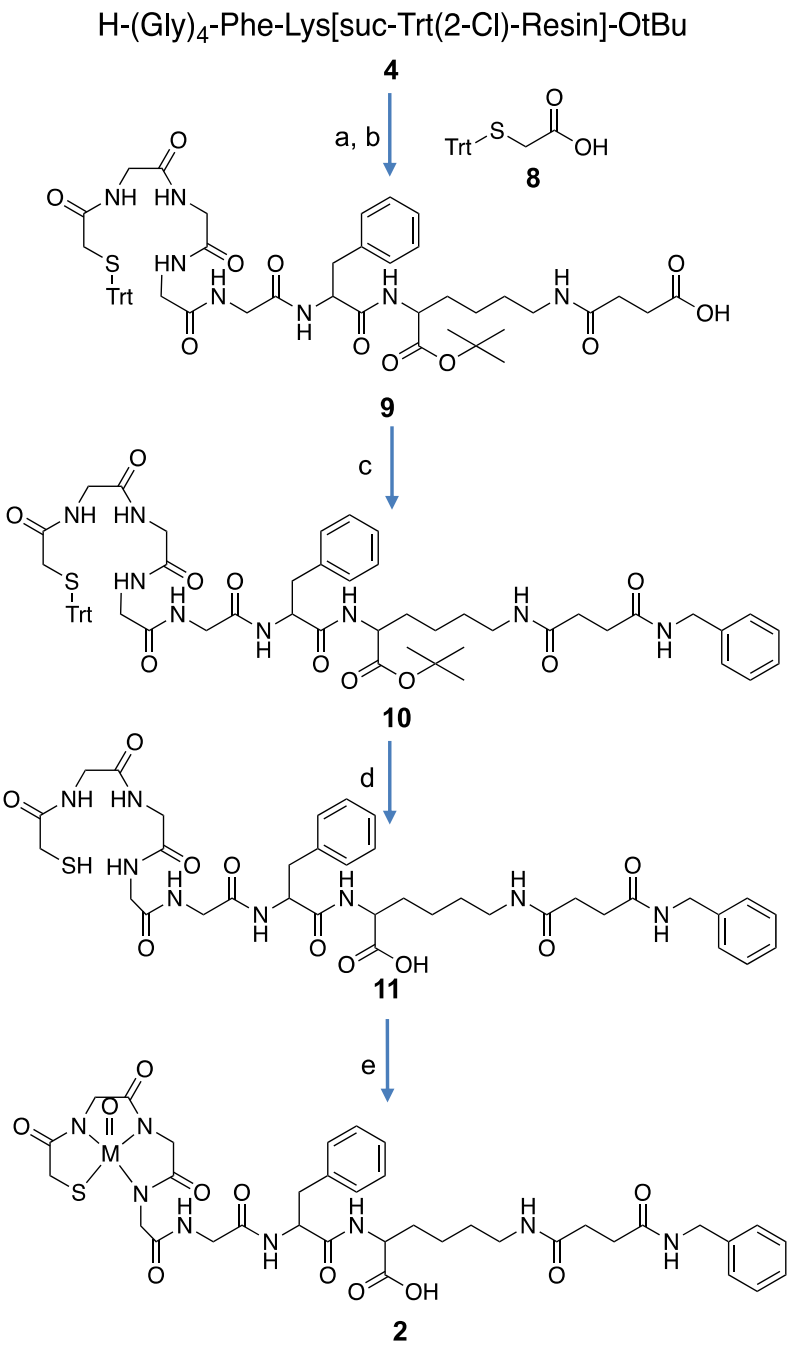


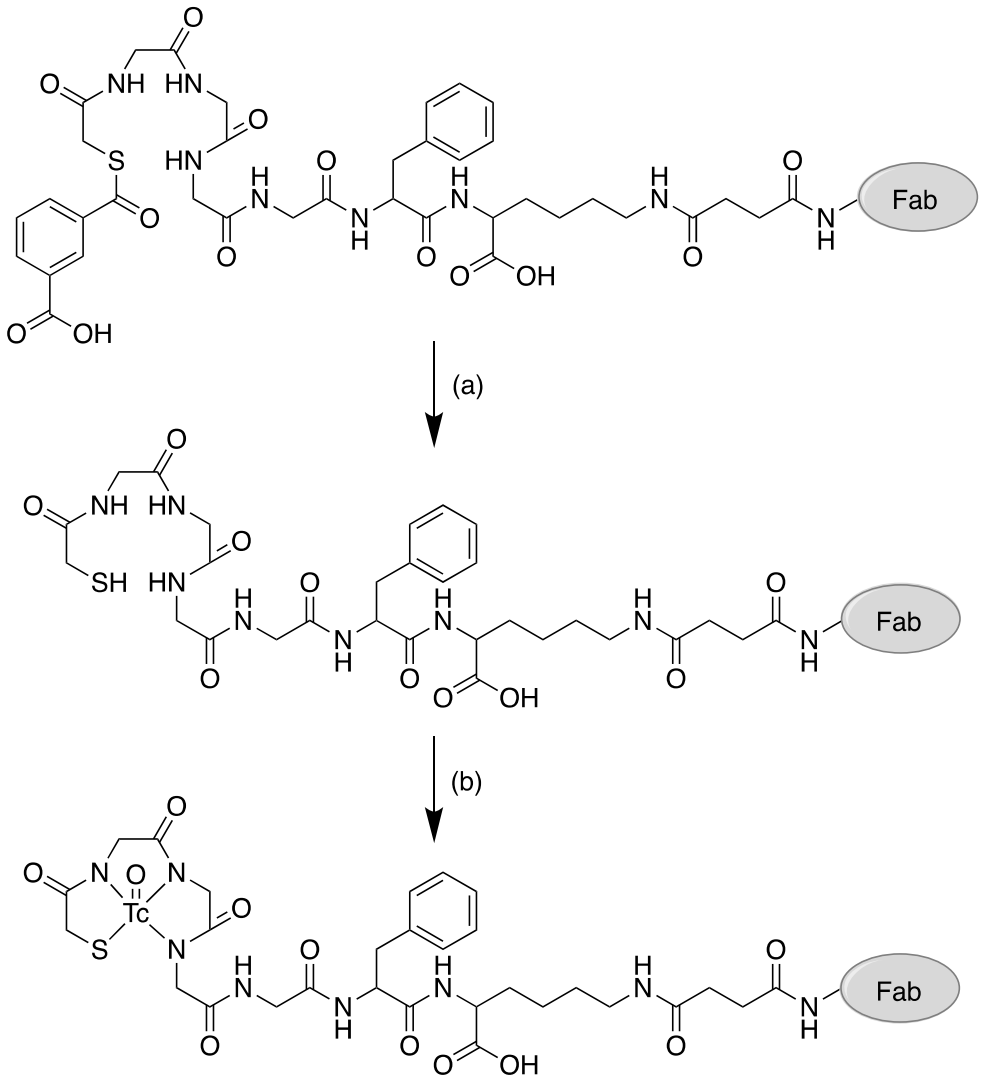
Figure S5. TLC profiles of [^{99m}Tc]Tc-MAG$_3$-GFK-suc-BA in the absence (left) and the presence of 1 mM captopril (middle) and MGTA (right). The tracer amount of [^{99m}Tc]Tc-MAG$_3$-GFK-suc-BA underwent partial decomposition by the presence of 1 mM concentration of thiol groups in captopril and MGTA.

Scheme S1. Synthetic procedure for M-MAG$_3$-GFK-suc-BA (M=^{99m}Tc or $^{185/187}$Re).



Reagents: (a) Compound **8** (2-[[3-(tert-butoxycarbonyl)benzoyl]thio]acetic acid), HOBt, DIC, (b) AcOH:TFE:CH_2Cl_2=3:1:6, (c) 2,3,5,6-tetrafluorophenol, DIC, (d) 10% anisole/TFA, (e) $ReOCl_4^-$ or ^{99m}Tc-glucarate.

Scheme S2. Synthetic procedure for [^{99m}Tc]Tc-MAG$_3$-GFK-Fab.



(a) 1 M NH_2OH (20% v/v), r.t., 10 min, (b) ^{99m}Tc-glucarate, 37 ˚C, 60 min

Table S1. Biodistribution of radioactivity after injection of ^{99m}Tc-MAG$_3$-GFK-Fab and ^{111}In-CHX-A”-DTPA-Fab in normal mice[a].

	Time after injection				
	10 min	30 min	1 h	3 h	6 h
	^{99m}Tc-MAG$_3$-GFK-Fab				
Blood	25.58 ± 1.22	17.81 ± 1.19	10.80 ± 0.80	4.09 ± 0.40	1.51 ± 0.17*
Liver	4.34 ± 0.36	3.67 ± 0.47	2.64 ± 0.18*	1.64 ± 0.12*	0.98 ± 0.44*
Spleen	3.20 ± 0.40	2.75 ± 0.38	1.90 ± 0.30*	0.85 ± 0.06*	0.48 ± 0.23*
Kidney	19.18 ± 2.55	22.91 ± 1.62*	17.00 ± 2.42*	6.81 ± 0.46*	3.12 ± 0.34*
Pancreas	0.88 ± 0.05*	1.12 ± 0.21	1.05 ± 0.05*	0.62 ± 0.06*	0.72 ± 0.59*
Heart	4.30 ± 0.89	5.00 ± 0.39	3.50 ± 0.31*	1.27 ± 0.11*	0.60 ± 0.03*
Lung	9.29 ± 1.82	8.84 ± 1.63	6.00 ± 0.89	2.09 ± 0.29*	0.93 ± 0.10*
Stomach[b]	0.49 ± 0.09*	0.60 ± 0.06*	0.69 ± 0.14	0.68 ± 0.14	0.65 ± 0.44
Intestine[b]	2.74 ± 0.23	5.53 ± 0.71*	7.76 ± 0.41*	15.66 ± 1.12*	20.63 ± 3.11*
Urine[b]					57.35 ± 3.97*
Feces[b]					0.04 ± 0.06*
Kidney/Blood	0.75 ± 0.11	1.24 ± 0.11*	1.57 ± 0.18*	1.68 ± 0.21*	2.07 ± 0.14*
	^{111}In-CHX-A”-DTPA-Fab				
Blood	25.16 ± 2.34	16.35 ± 1.71	11.68 ± 1.81	3.79 ± 0.60	1.26 ± 0.17
Liver	4.63 ± 0.53	4.26 ± 0.46	4.16 ± 0.72	4.93 ± 0.77	4.05 ± 0.53
Spleen	3.44 ± 0.58	3.25 ± 0.47	3.05 ± 0.69	3.77 ± 0.70	3.05 ± 0.49
Kidney	19.12 ± 2.94	31.87 ± 3.03	46.09 ± 6.06	56.33 ± 6.80	41.78 ± 5.28
Pancreas	1.02 ± 0.09	1.17 ± 0.16	1.33 ± 0.23	1.49 ± 0.48	1.94 ± 0.48
Heart	4.92 ± 0.55	5.11 ± 0.54	4.82 ± 0.25	3.85 ± 0.32	3.02 ± 0.56
Lung	8.62 ± 1.63	7.33 ± 1.81	5.07 ± 0.45	3.08 ± 0.54	1.63 ± 0.08

Stomach[b]	0.37 ± 0.03	0.46 ± 0.07	0.58 ± 0.07	0.66 ± 0.13	0.61 ± 0.12
Intestine[b]	2.48 ± 0.28	3.53 ± 0.16	4.06 ± 0.32	4.96 ± 0.49	5.33 ± 1.13
Urine[b]					20.97 ± 4.46
Feces[b]					0.29 ± 0.17
Kidney/Blood	0.76 ± 0.11	1.95 ± 0.14	4.04 ± 0.90	15.01 ± 1.74	33.69 ± 6.06

[a]Results (means ± SD of five animals each point) were expressed as % injected dose per gram of wet tissue. [b]Expressed as % injected dose per tissue. Significance determined by Welch's test. * $P<0.05$ compared to ^{111}In-CHX-A"-DTPA-Fab