**Supporting information**

**Gallium-67/68-labeled antibody fragments for immuno-SPECT/PET show low renal radioactivity levels without loss of tumor uptake**

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**Synthetic methods of ligands**

**General**

Analytical reversed phase HPLC (RP-HPLC) was performed with a Cosmosil 5C18-AR-300 column (4.6 mm × 150 mm, Nacalai Tesque Inc., Kyoto, Japan) at a flow rate of 1 mL/min using a gradient mobile phase starting from 95% A (0.1% aqueous trifluoroacetic acid (TFA)) and 5% B (acetonitrile with 0.1% TFA) to 70% A and 30% B in 20 min, followed by a change to 0% A and 100% B in 40 min. Preparative RP-HPLC was performed with a Cadenza 5CD-C18 column (20 mm × 150 mm, Imtakt, Kyoto) connected to a Cadenza 5CD-C18 guard column (10 mm × 8 mm, Imtakt) at a flow rate of 5 mL/min with a gradient mobile phase starting at 90% A and 10% B to 20% A and 80% B in 30 min, followed by a change to 0% A and 100% B in 40 min. Size-exclusion HPLC (SE-HPLC) was performed with a Cosmosil 5Diol-300-II column (7.5 × 600 mm, Nacalai Tesque Inc.) connected to a Cosmosil Diol-300-II guard column (7.5 mm × 50 mm, Nacalai Tesque Inc.) eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1 mL/min. The eluent was monitored with an online UV/Visible single beam detector at 254 nm or 280 nm (L-7405, Hitachi Co. Ltd., Tokyo, Japan) coupled to a NaI(Tl) radioactivity detector (Gabi Star, Raytest, Strubenhardt, Germany). Otherwise, each eluent was collected with a fraction collector (Frac-920, GE Healthcare Japan, Tokyo) at 0.5-min intervals, and the radioactivity counts in each fraction were determined with an automated gamma well counter (Wizard 3, PerkinElmer Japan, Yokohama, Japan). TLC analyses were performed with RP18 plates (Silica gel 60 RP-18 F254S, Merck Ltd., Tokyo) developed with 0.1 M ammonium acetate/methanol (1/1). Cellulose acetate electrophoresis (CAE, ADVANTEC SELECA-V, Toyo Roshi Ltd., Tokyo) strips were run in a veronal buffer (pH 8.5, *I*=0.06) at a constant current of 1 mA/cm for 90 min. Radioactivity was measured using a γ-miniGITA TLC Scanner (Raytest) and an automated gamma well counter. Mass spectrometry and high-resolution mass spectrometry (HRMS) were performed using a JMS-T100LP (JEOL Ltd., Tokyo). 1H-NMR spectra were recorded on a JEOL ECS-400 spectrometer (JEOL Ltd., Tokyo). NOTA-MI-Mal, NOTA-conjugated methionine or lysine (NOTA-Met, NOTA-Lys), and gallium-labeled NOTA-Met or NOTA-Lys were synthesized as described previously.(1)

**Synthesis of Boc-Met-Val-Lys-OH**

Boc-Met-Val-Lys-OH was synthesized by manual Fmoc solid-phase peptide synthesis using Cl-Trt(2-Cl)-Resin (Watanabe Chemical Industries, Ltd., Hiroshima, Japan, 195 mg, 0.313 mmol) as the solid phase and the following protected amino acids Fmoc-Lys(Dde)-OH, Fmoc-Val-OH, and Boc-Met-OH. The peptide chain was constructed manually according to the published method consisting of (i) 2 h of coupling of the protected amino acid derivative (2.8 equiv.) in the presence of *N,N’*-diisopropylcarbodiimide (DIC, 2.8 equiv.) and 1-hydroxybenzotriazole/H2O (HOBt, 2.8 equiv.) in *N,N’*-dimethylformamide (DMF, 3 mL) and (ii) 20 min of deprotection with 20% piperidine-DMF. After constructing the peptide sequence, the peptide resin was treated with 2% hydrazine/DMF (3 mL) for 1 h to cleave the Dde group of Lys. The peptide resin was then treated with a mixture of acetic acid/2,2,2-trifluoroethanol/dichloromethane (3/1/6; 2 mL) for 2 h to cleave the assembled peptide from the resin. After filtration, the filtrate was treated with diethyl ether (3 mL) to precipitate Boc-Met-Val-Lys-OH as a white solid (84 mg, 89.5%). 1H-NMR (CD3OD) δ: 0.97 [6H, m, C*H3*], 1.44 [9H, s, O*tBu*], 1.88 [12H, m, C*H2*, C*H*, SC*H3*], 2.52 [2H, m, C*H2*NH2], 2.91 [2H, t, SC*H2*], 4.51 [3H, m, C*H*CO]; ESI-MS m/z [M + H]+ 477, Found 477. m.p. 203-205 ˚C.

**Synthesis of Boc-Val-Lys(Mal)-OH**

*N*-(Methoxycarbonyl)maleimide (NMCM) (3 mg, 20 µmol) was added to a solution of Boc-Met-Val-Lys-OH (6.6 mg, 13.9 µmol) in 100 µL of saturated aqueous NaHCO3 at 0 ˚C with vigorous stirring. After 2 h, the pH of the solution was adjusted to 3 with 5% citric acid at the same temperature. The solution was extracted with chloroform (5 mL × 3) and then dried over anhydrous Na2SO4. After removing the solvent *in vacuo*, Boc-Val-Lys-(Mal)-OH was obtained as a white solid (7.3 mg, 94.1%). 1H-NMR (CDCl3) δ: 0.93 [6H, m, C*H3*], 1.38 [2H, m, C*H2*], 1.44 [9H, s, *Boc*], 1.59 [2H, *CH2*CH2-maleimide], 1.81 [1H, m, C*H*], 2.01 [2H, m, *CH2*CHCO], 2.19 [5H, m, *CH2*CH2SCH3, SC*H3*], 2.54 [2H, t, SC*H2*], 3.51 [2H, m, C*H2*-maleimide], 4.25 [3H, m, C*H*CO], 6.72 [2H, s, *maleimide*]; ESI-MS m/z [M + H]+ 557, Found 557. m.p. 124-125 ˚C.

**Synthesis of Met-Val-Lys(Mal)-OH**

Boc-Met-Val-Lys(Mal)-OH (7.3 mg, 13 µmol) was dissolved in 4 *N* HCl/ethylacetate (ca. 1 mL), and the mixture was stirred at room temperature for 1 h. After removing the solvent *in vacuo*, the HCl salt of Met-Val-Lys(Mal)-OH was obtained as a white solid (6 mg, 92%). 1H-NMR (CDCl3) δ: 1.00 [6H, m, C*H3*], 1.29 [2H, m, C*H2*], 1.39 [2H, m, C*H2*CH2-maleimide], 1.60 [1H, m, C*H*], 2.07 [7H, m, C*H2*CHO, C*H2*CH2SCH3, SC*H3*], 2.53 [2H, t, SC*H2*], 3.52 [2H, m, C*H2*-maleimide], 4.14 [3H, m, C*H*CO], 6.82 [2H, s, *maleimide*]; ESI-MS m/z [M + H]+ 457, Found 457. m.p. 143-145 ˚C.

**Synthesis of NOTA-Met-Val-Lys(Mal)-OH (NOTA-MVK-Mal)**

The HCl salt of Met-Val-Lys(Mal)-OH (5 mg, 10.2 µmol) was dissolved in dry DMF (100 µL), and triethylamine (6 µL) was added. To the solution was added SCN-Bn-NOTA chelate (7 mg, 16 µmol), and the mixture was stirred for 2 h at room temperature. After diluting with water (× 10), the compound was purified with preparative RP-HPLC to obtain NOTA-MVK-Mal as a white solid (2.8 mg, 28.2%). 1H-NMR (D2O) δ: 0.93 [6H, m, C*H3*], 1.29 [2H, m, C*H2*], 1.54 [2H, m, C*H2*CH2-maleimide], 1.81 [2H, m, C*H2*CHCO], 2.07 [6H, m, C*H*, C*H2*CH2SCH3, SC*H3*], 2.54 [2H, m, SC*H2*], 3.23 [21H, m, C*H2*-maleimide, C*H2*, C*H*, C*H2*COOH], 4.19 [3H, m, C*H*CO], 6.78 [2H, s, *maleimide*], 7.30 [4H, m, *Ar-H*]; ESI-MS m/z [M + H]+ 907, Found 907; HRMS m/z [M + H]+ calculated for C40H59N8O12S2 907.36938, observed 907.36338. m.p. 131-133 ˚C.

**Synthesis of Met-Val-Lys-Bzo**

Met-Val-Lys-Bzo was synthesized using methods similar to those described above using Fmoc-Met-OH and Fmoc-Lys(Bzo) instead of Boc-Met-OH and Fmoc-Lys(Dde)-OH. The Met-Val-Lys-Bzo was obtained as a white solid (9.7 mg, 69%). 1H-NMR (D2O) δ:0.91 [6H, m, C*H3*], 1.63 [6H, m, C*H2*], 2.02 [6H, m, SCH2C*H2*, COCHC*H*, SC*H3*], 2.47 [2H, m, SC*H2*], 3.34 [2H, m, NHC*H2*], 4.11 [3H, m, COC*H*], 7.61 [5H, m, Ar-*H*]; ESI-MS m/z [M + H]+ 481, Found 481. M.p. 179-180 ˚C.

**Synthesis of NOTA-Met-Val-Lys-Bzo (NOTA-MVK-Bzo)**

The SCN-Bn-NOTA (5.6 mg, 12.5 µmol) was dissolved in 0.1 M borate buffer (pH 9.0) adjusted to pH at 8-9 with 0.1 N NaOH aq, after which Met-Val-Lys-Bzo (4.0 mg, 8.3 µmol) was added to the solution and stirred at room temperature for 2 h. The desired compound was purified by preparative RP-HPLC and NOTA-MVK-Bzo was obtained as a white solid (2.5 mg, 32%). 1H-NMR (DMSO-D6) δ:0.88 [6H, m, C*H3*], 1.46 [6H, m, C*H2*], 2.04 [6H, m, SCH2C*H2*, COCHC*H*, SC*H3*], 3.35 [26H, m, C*H2*, C*H*, SC*H2*, COC*H*], 7.80 [9H, m, Ar-*H*]; ESI-MS m/z [M + H]+ 931, Found 931. HRMS m/z [M + H]+ calculated for C43H63N8O11S2 931.40577, Found 931.41015, M.p. 146-148 ˚C.

**Synthesis of Ga-NOTA-MVK-Bzo**

NOTA-MVK-Bzo (4.2 mg, 4.51 µmol) was added to a solution of Non-radioactive GaCl3 (1.2 mg, 6.77 µmol) in 0.25 M acetate buffer (pH 5.5, 50 µL). After stirring for 1 h, Ga-NOTA-MVK-Bzo was purified by preparative RP-HPLC as a white solid (1 mg, 22.3%). ESI-MS m/z [M - H]- 995, Found 995.

**Preparation of 67Ga-NOTA-MVK-Bzo**

A 10 µL solution of 67GaCl3 in 0.05 *N* HCl was mixed with 0.25 M acetate buffer (pH 5.5, 10 µL). After 5 min, a solution of NOTA-MVK-Bzo (2 × 10-4 M, 10 µL) in 0.1 M acetate buffer (pH 5.5) was added, and the mixture was incubated at 60 ˚C for 1 h. Radiochemical purity was analyzed by RP-HPLC equipped with an on-line UV/Vis detector and radioactivity detector. The authenticity of the radiochemical species was verified by co-injection of the reaction solution with a characterized non-radioactive Ga-NOTA-MVK-Bzo standard.

**Preparation of NOTA-Fab conjugates**

A solution of Fab (200 µL, 2 mg/mL) in well-degassed 0.16 M borate buffer (pH 8.0) containing 2 mM EDTA was reacted with 7.5 µL (2.5 µL × 3) of 2-iminothiolane (2-IT) solution (2 mg/mL) prepared in the same buffer. After gentle agitation of the reaction mixture at 37 ˚C for 30 min, excess 2-IT was removed by a centrifuged column procedure using Sephadex G-50 fine (GE Healthcare Japan) equilibrated and eluted with 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA. Aliquots of the solution were sampled for estimation of the number of thiol groups with 2,2’-dipyridyl disulfide (DPS). The filtrate (95 µL, 1.6 mg/mL) was then added to a solution of NOTA-MVK-Mal or NOTA-MI-Mal (50 mg/mL, 1.25 µL) in 0.1 M phosphate buffer (pH 6.0). After gentle agitation of the reaction mixture at 37 ˚C for 1 h, excess NOTA-MVK-Mal or NOTA-MI-Mal was removed by a centrifuged column using Sephadex G-50 fine, equilibrated, and eluted with 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA. Aliquots of the filtrate containing the NOTA-Fab conjugates were then sampled to estimate the number of thiol groups with DPS. To cap and alkylate unreacted thiol groups, 12.5 µL of iodoacetamide (10 mg/mL) in 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA was then added, and the reaction mixture was further incubated at 37 ˚C for 1 h. The NOTA-derivatized Fab-conjugates were finally purified by the centrifuged column procedure using Sephadex G-50 fine equilibrated and eluted with 0.25 M acetate buffer (pH 5.5).

**Preparation of NOTA-SCN-conjugated Fab (NOTA-Fab)**

The Fab fragment (5 mg/mL, 100 µL) in 0.1 M borate buffer (pH 9.0) was mixed with SCN-Bn-NOTA (2.3 µL, 5 mg/mL in DMF). After incubating at room temperature for 12 h, the conjugate was purified by the centrifuged column procedure using Sephadex G-50 fine, equilibrated and eluted with 0.25 M acetate buffer (pH 5.5).

**Preparation of 67Ga-labeled Fab Fragments.**

A 5 µL solution of 67GaCl3 (0.05 *N* HCl) was added to 0.25 M acetate buffer (pH 5.5, 5 µL). After 5 min, each NOTA-Fab conjugate (10 µL, 2 mg/mL, 0.25 M acetate buffer pH 5.5) was added to the solution, and the solution was gently incubated at 37 ˚C for 1 h. A 20 µL solution of 20 mM EDTA was then added, and the mixture was incubated for 30 min at the same temperature. Each 67Ga-labeled Fab fragment was purified by a centrifuged column procedure using Sephadex G-50 fine, equilibrated and eluted with D-PBS. Radiochemical yields and purities of each 67Ga-labeled NOTA-Fab conjugate were determined by RP-TLC, CAE, and SE-HPLC and were obtained in over 95% radiochemical yields and purities. For *in vivo* animal studies, 67Ga-labeled NOTA-Fab conjugates were diluted with D-PBS to the desired concentrations and activities.

**Table S1.** Biodistribution of radioactivity in mice after injection of 67Ga-NOTA-Fab, 67Ga-NOTA-MI-Fab and 67Ga-NOTA-MVK-Fab.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Tissue radioactivity is expressed as %ID/g [for each group, n=5; results are reported as mean ± SD] | | | | | | | | | | | | | |
|  | Time after injection | | | | | | | | | | | | |
|  | 10 min | | 1 h | | | | 3 h | | | 6 h | | 24 h | |
|  | 67Ga-NOTA-Fab | | | | | | | | | | | | |
| Blood | 22.93 | ± 2.37 | | 8.13 | ± 0.64 | | 2.91 | ± 0.46 | | 1.54 | ± 0.29 | 0.13 | ± 0.02 |
| Liver | 3.45 | ± 0.22 | | 2.21 | ± 0.17 | | 1.79 | ± 0.24 | | 1.84 | ± 0.22 | 0.81 | ± 0.13 |
| Kidney | 22.51 | ±3.60 | | 46.90 | ± 4.01 | | 60.43 | ± 12.68 | | 55.76 | ± 17.26 | 17.27 | ± 3.45 |
| Spleen | 2.73 | ± 0.30 | | 1.25 | ± 0.16 | | 0.81 | ± 0.12 | | 0.70 | ± 0.11 | 0.50 | ± 0.07 |
| Stomach\* | 0.51 | ± 0.20 | | 0.51 | ± 0.04 | | 0.42 | ± 0.06 | | 0.31 | ± 0.04 | 0.19 | ± 0.13 |
| Intestine\* | 2.47 | ± 0.41 | | 3.18 | ± 0.26 | | 2.48 | ± 0.44 | | 2.73 | ± 0.59 | 1.61 | ± 0.49 |
| Urine\* |  |  | |  | |  |  |  | | 33.98 | ± 9.21 | 72.27 | ± 3.65 |
| Feces\* |  |  | |  | |  |  |  | | 0.06 | ± 0.06 | 3.47 | ± 0.70 |
|  | 67Ga-NOTA-MI-Fab | | | | | | | | | | | | |
| Blood | 24.96 | ± 1.63 | | 10.97 | ± 1.22 | | 3.77 | ± 0.21 | | 1.59 | ± 0.15 | 0.15 | ± 0.01 |
| Liver | 3.89 | ± 0.40 | | 3.00 | ± 0.41 | | 2.13 | ± 0.43 | | 1.78 | ± 0.22 | 0.74 | ± 0.21 |
| Kidney | 22.21 | ± 2.40 | | 43.94 | ± 3.71 | | 32.44 a | ± 2.36 | | 23.38 a | ± 2.11 | 10.75 a | ± 2.20 |
| Spleen | 3.18 | ± 0.46 | | 2.67 | ± 0.46 | | 1.65 | ± 0.54 | | 1.02 | ± 0.20 | 0.65 | ± 0.26 |
| Stomach\* | 0.34 | ± 0.04 | | 0.47 | ± 0.07 | | 0.42 | ± 0.03 | | 0.43 | ± 0.16 | 0.75 | ± 0.57 |
| Intestine\* | 1.67 | ± 0.34 | | 2.98 | ± 0.52 | | 4.31 | ± 0.44 | | 8.77 | ± 2.12 | 3.12 | ± 1.42 |
| Urine\* |  |  | |  |  | |  | |  | 46.23 | ± 2.33 | 68.91 | ± 4.91 |
| Feces\* |  |  | |  |  | |  |  | | 0.01 | ± 0.00 | 6.51 | ± 2.72 |
|  | 67Ga-NOTA-MVK-Fab | | | | | | | | | | | | |
| Blood | 26.04 | ± 2.21 | | 12.63 | ± 1.19 | | 5.50 | | ± 0.41 | 2.45 | ± 0.39 | 0.23 | ± 0.04 |
| Liver | 3.43 | ± 0.28 | | 2.51 | ± 0.23 | | 1.72 | | ± 0.16 | 1.27 | ± 0.24 | 0.27 | ± 0.07 |
| Kidney | 15.38a,b | ± 2.06 | | 17.90 a,b | ± 1.85 | | 12.33 a,b | | ± 1.25 | 8.43 a,b | ± 1.30 | 1.48 a,b | ± 0.18 |
| Spleen | 3.34 | ± 0.24 | | 2.44 | ± 0.24 | | 1.34 | | ± 0.19 | 0.78 | ± 0.16 | 0.22 | ± 0.06 |
| Stomach\* | 0.34 | ± 0.02 | | 0.62 | ± 0.06 | | 0.54 | | ± 0.09 | 0.44 | ± 0.12 | 0.16 | ± 0.14 |
| Intestine\* | 2.26 | ± 0.39 | | 4.33 | ± 0.25 | | 5.65 | | ± 1.05 | 10.19 | ± 1.19 | 1.44 | ± 0.76 |
| Urine\* |  |  | |  | |  |  |  | | 55.65 | ± 10.40 | 72.51 | ± 6.01 |
| Feces\* |  |  | |  | |  |  |  | | 0.29 | ± 0.36 | 16.06 | ± 2.24 |

\* Expressed as %ID

Significance determined by one-way analysis of variance followed by Tukey’s multiple-comparison test.

*a P*<0.05 compared to 67Ga-NOTA-Fab

*b P*<0.05 compared to 67Ga-NOTA-MI-Fab

**Table S2.** Biodistribution of radioactivity in nude mice bearing SY cells after 3 h injection of 67Ga-NOTA-Fab, 67Ga-NOTA-MI-Fab and 67Ga-NOTA-MVK-Fab.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Tissue radioactivity is expressed as %ID/g [for each group, n=3-5; results are reported as mean ± SD] | | | | | | |
|  | 67Ga-NOTA-Fab | | 67Ga-NOTA-MI-Fab | | 67Ga-NOTA-MVK-Fab | |
| Blood | 2.33 | ± 0.12 | 3.84 | ± 0.27 | 4.74 | ± 0.26 |
| Liver | 3.16 | ± 0.40 | 4.15 | ± 0.28 | 2.87 | ± 0.36 |
| Kidney | 96.63 | ± 8.46 | 64.11a | ± 8.23 | 16.52a,b | ± 1.64 |
| Spleen | 2.66 | ± 0.40 | 3.89 | ± 0.76 | 1.85 | ± 0.12 |
| Stomach\* | 0.24 | ± 0.03 | 0.26 | ± 0.05 | 0.37 | ± 0.12 |
| Intestine\* | 2.14 | ± 0.12 | 4.21 | ± 0.88 | 6.28 | ± 0.77 |
| Tumor | 8.59 | ± 1.84 | 10.35 | ± 1.49 | 10.39 | ± 0.84 |

\* Expressed as %ID

Significance determined by one-way analysis of variance followed by Tukey’s multiple-comparison test.

*a P*<0.05 compared to 67Ga-NOTA-Fab

*b P*<0.05 compared to 67Ga-NOTA-MI-Fab

**Table S3.** Stability of 67Ga-labeled Fab fragments in murine serum.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Percent of intact 67Ga-labeled Fab fragment a | | |
| Time (h) | 67Ga-NOTA-Fab | 67Ga-NOTA-MI-Fab | 67Ga-NOTA-MVK-Fab |
| 1 | 99.18 ±0. 21 | 98.36 ± 0.20 | 97.05 ± 0.35 |
| 3 | 99.42 ± 0.08 | 98.20 ± 0.14 | 97.02 ± 0.16 |
| 6 | 98.80 ± 0.16 | 98.01 ± 0.22 | 96.65 ± 0.34 |
| 24 | 98.86 ± 0.20 | 96.72 ± 0.29 | 95.58 ± 0.44 |

a Results are expressed as mean ± SD of three experiments.

**Scheme S1. Synthetic procedure for NOTA-MVK-Mal**

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**Reagents:** (a) i) Fmoc-L-Lys(Dde)-OH, ii) 20% piperidine/DMF; (b) i) Fmoc-L-Val-OH, DIC, HOBt, ii) 20% piperidine/DMF; (c) Boc-L-Met-OH, DIC, HOBt; (d) 2% hydradine/DMF; (e) acetic acid : 2,2,2-trifluoroethanol : dichloromethane = 3 : 1 : 6 (v/v/v); (f) NMCM, Sat. NaHCO3 aq.; (g) 4 *N* HCl/ethylacetate; (h) SCN-Bn-NOTA, TEA, DMF.

**Scheme S2.** Synthetic procedure for NOTA-MVK-Bzo.



**Reagents:** (a) i) Fmoc-L-Lys(Bzo)-OH, ii) 20% piperidine/DMF; (b) i) Fmoc-L-Val-OH, DIC, HOBt, ii) 20% piperidine/DMF; (c) Fmoc-L-Met-OH, DIC, HOBt; (d) 2% hydrazine/DMF; (e) SCN-Bn-NOTA, 0.1 M borate buffer (pH 9.0).

Reference

1. Wu C, Jagoda E, Brechbiel M, Webber KO, Pastan I, Gansow O*, et al.* Biodistribution and catabolism of Ga-67-labeled anti-Tac dsFv fragment. Bioconjugate Chem **1997**;8(3):365-9.