

# Supplementary Information

## Preclinical Evaluation of <sup>177</sup>Lu-Labeled PSMA and FAP Bispecific Tracer for Prostate Cancer Targeted Radionuclide Therapy

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## 1. Chemical Synthesis

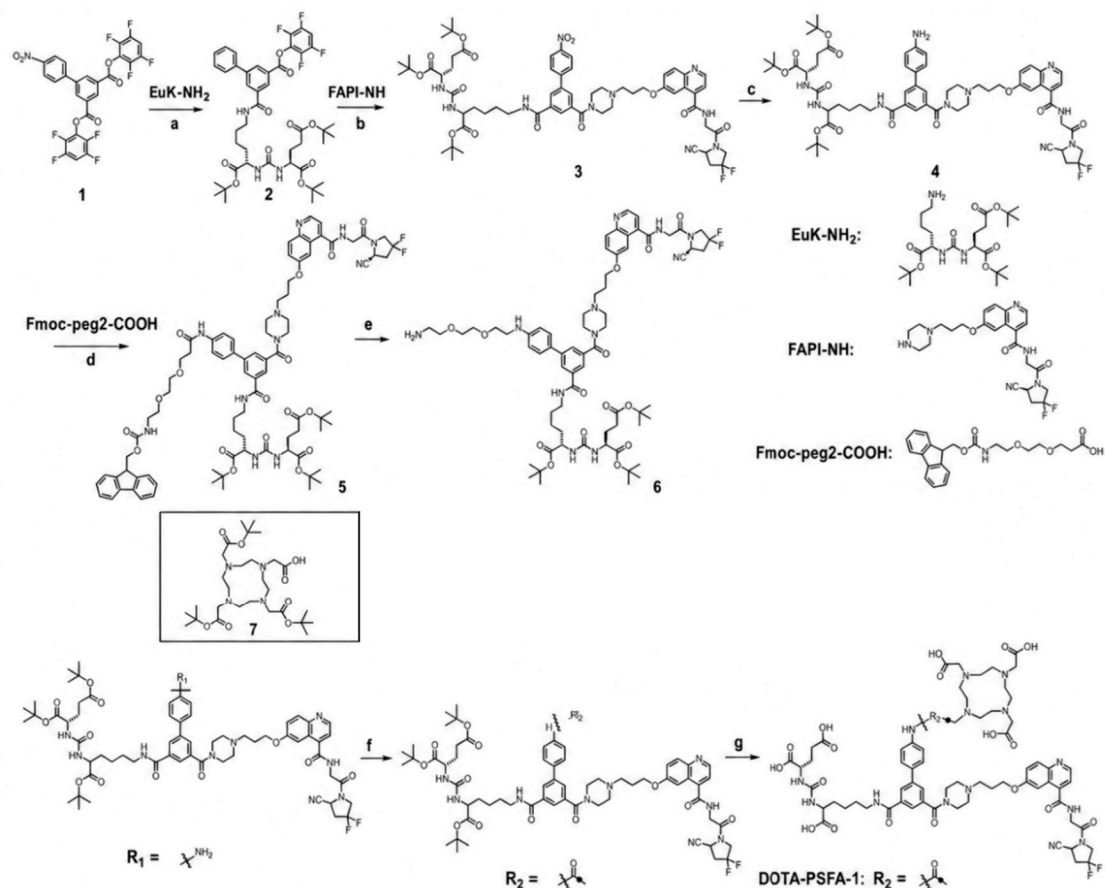


Figure S1. Synthetic route of the precursors. Reagents and Conditions:

- (a) CH<sub>2</sub>Cl<sub>2</sub>, EuK-NH<sub>2</sub>, Et<sub>3</sub>N, r.t., 40.0%;
- (b) CH<sub>2</sub>Cl<sub>2</sub>, FAPI-NH, Et<sub>3</sub>N, r.t., 75.4%;
- (c) 4,4'-bipyridine (0.75 mM in DMF), tetrahydroxydiborane, r.t., 86.7%;
- (d) iPrOH, Fmoc-PEG<sub>2</sub>-COOH, DMTMM, 40 °C, 91.8%;
- (e) CH<sub>2</sub>Cl<sub>2</sub>, piperidine, r.t., 83.8%;
- (f) iPrOH, DMTMM, 40 °C, for **4**, 42.0-78.6%; CH<sub>2</sub>Cl<sub>2</sub>, DOTA-tris(tBu) ester, EDCI, HOBT, DIPEA, DMAP, for **6**, 34.2-71.7%;
- (g) CH<sub>2</sub>Cl<sub>2</sub>/CF<sub>3</sub>COOH, r.t., 65.2-93.2%.

## 2. Characterization

DOTA-PSFA-1 (5.0 mg) was dissolved in 260  $\mu\text{L}$  of NaOAc solution (1 M) in a glass vial. HCl solution (2.0 mL, 0.1 M) and  $\text{LuCl}_3$  (10 equiv.) were subsequently added. The vial was crimp-sealed, and the reaction mixture was heated at 100  $^\circ\text{C}$  for 15 min. After cooling to room temperature, the mixture was diluted to 5 mL with  $\text{H}_2\text{O}$  and passed through a 0.22  $\mu\text{m}$  membrane filter (Whatman, UK). The crude product was purified by preparative HPLC using an SCL-20AVP system (Shimadzu, Japan). The purified fraction was collected and lyophilized to afford  $[\text{natLu}]\text{Lu-DOTA-PSFA-1}$  as a white flocculent solid in 8% yield. Preparative HPLC was performed on a Venusil MP C18 reversed-phase column (10  $\mu\text{m}$ , 10  $\times$  250 mm; Bonna-Agela Technologies, China) at a flow rate of 4.0 mL/min. The mobile phase consisted of  $\text{H}_2\text{O}$  containing 0.1% TFA (solvent A) and acetonitrile (solvent B), with an isocratic A/B ratio of 80:20 (v/v).  $[\text{natLu}]\text{Lu-DOTA-PSFA-1}$ : HRMS (ESI-)  $m/z$ : 527.4943 (calcd for  $[\text{M} - 3\text{H}]^{3-}$ , 527.4960).

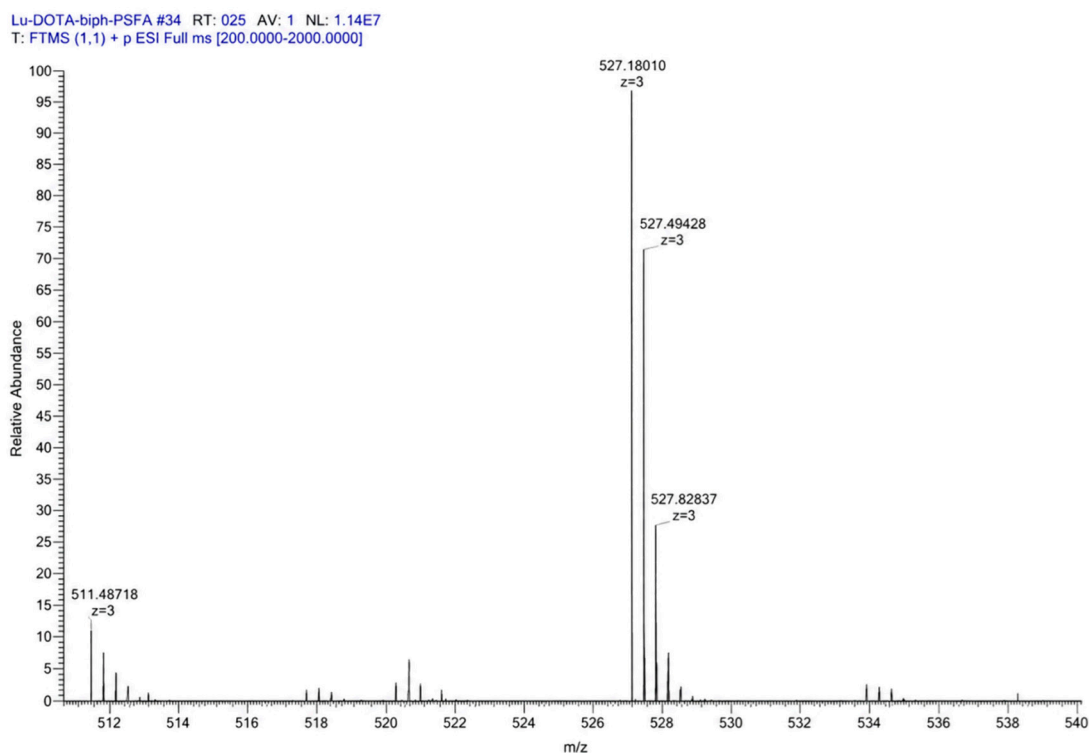


Figure S2. HRMS spectrum of compound  $[\text{natLu}]\text{Lu-DOTA-PSFA-1}$ .

### 3. Western Blot

PSMA and FAP expression in 22Rv1 and U87MG tumor tissues, respectively, was evaluated by Western blot. Briefly, freshly collected tumor tissues were washed two to three times with ice-cold PBS to remove residual blood, minced into small pieces, and transferred into homogenization tubes containing two 4-mm grinding beads. RIPA lysis buffer supplemented with protease inhibitors was added at approximately 10 times the tissue volume, and the tissues were mechanically homogenized. The homogenates were incubated on ice for 30 min with vortexing every 5 min and subsequently centrifuged at 12,000 rpm for 10 min at 4 ° C. The supernatants were collected as total protein extracts.

Protein concentrations were determined using a BCA protein assay kit (Servicebio, Wuhan, China). Protein samples were mixed with 5× reducing SDS-PAGE loading buffer at a 4:1 ratio and denatured in a boiling water bath for 15 min. The proteins were separated by 10% SDS-PAGE and transferred onto 0.45- μ m polyvinylidene difluoride (PVDF) membranes at a constant current of 350 mA for 30 min.

The membranes were blocked with 5% non-fat milk for 30 min at room temperature and incubated overnight at 4 ° C with primary antibodies against PSMA (Servicebio, GB115710; 1:1000), FAP (Proteintech, 11779-1-AP; 1:1000), or β -actin (Servicebio, GB15003; 1:5000). After primary antibody incubation, the membranes were washed three times with TBST for 5 min each and subsequently incubated with HRP-conjugated goat anti-rabbit IgG at room temperature for 30 min. After three additional washes with TBST, immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection system.

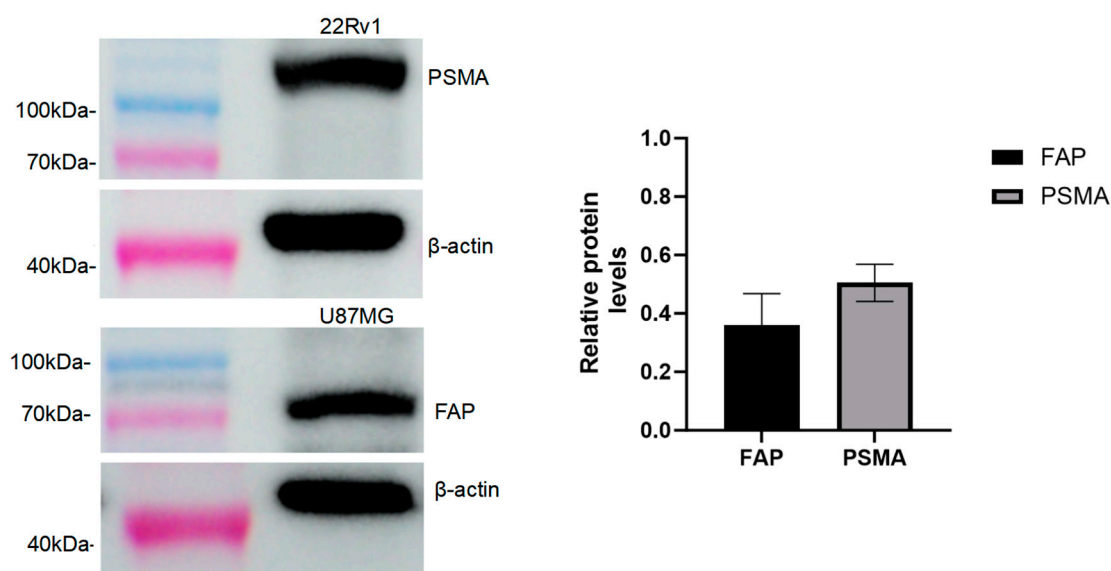


Figure S3. Western blot analysis of PSMA and FAP expression in 22Rv1 and U87MG xenograft tissues, respectively. Protein levels were normalized to β -actin and are presented as mean ± SD (n = 3).

#### 4. Biodistribution

**Table S1.** Biodistribution results of [ $^{177}\text{Lu}$ ]Lu-PSFA-1 in 22Rv1 and U87MG tumor-bearing mice at 1, 4, 8, 24, 48 and 96 h p.i., each mouse received 740 kBq. Data are expressed as %ID/g and presented as mean  $\pm$  SD (n = 4).

| Organ       | 1 h              | 4 h              | 8 h              |
|-------------|------------------|------------------|------------------|
| Blood       | 2.24 $\pm$ 0.17  | 0.75 $\pm$ 0.09  | 0.46 $\pm$ 0.04  |
| Brain       | 0.09 $\pm$ 0.02  | 0.05 $\pm$ 0.01  | 0.04 $\pm$ 0.01  |
| Liver       | 0.59 $\pm$ 0.03  | 0.52 $\pm$ 0.07  | 0.58 $\pm$ 0.07  |
| Spleen      | 0.75 $\pm$ 0.63  | 0.41 $\pm$ 0.01  | 0.35 $\pm$ 0.15  |
| Kidney      | 10.64 $\pm$ 1.13 | 5.80 $\pm$ 0.97  | 6.74 $\pm$ 0.91  |
| Stomach     | 1.74 $\pm$ 1.09  | 0.36 $\pm$ 0.03  | 0.33 $\pm$ 0.05  |
| Intestine   | 1.82 $\pm$ 0.73  | 0.92 $\pm$ 0.59  | 0.79 $\pm$ 0.16  |
| Heart       | 0.72 $\pm$ 0.02  | 0.29 $\pm$ 0.05  | 0.26 $\pm$ 0.02  |
| Lung        | 1.43 $\pm$ 0.16  | 0.54 $\pm$ 0.07  | 0.53 $\pm$ 0.19  |
| Bone        | 4.09 $\pm$ 0.51  | 1.81 $\pm$ 0.60  | 1.93 $\pm$ 0.30  |
| Muscle      | 1.35 $\pm$ 0.26  | 0.48 $\pm$ 0.04  | 0.53 $\pm$ 0.03  |
| U87MG tumor | 23.93 $\pm$ 5.02 | 11.61 $\pm$ 1.95 | 10.02 $\pm$ 2.43 |
| 22Rv1 tumor | 7.57 $\pm$ 0.50  | 4.71 $\pm$ 0.25  | 4.51 $\pm$ 0.98  |

| Organ       | 24 h              | 48 h              | 96 h              |
|-------------|-------------------|-------------------|-------------------|
| Blood       | 0.13 $\pm$ 0.01   | 0.05 $\pm$ 0.01   | 0.04 $\pm$ 0.02   |
| Brain       | 0.030 $\pm$ 0.004 | 0.02 $\pm$ 0.01   | 0.021 $\pm$ 0.003 |
| Liver       | 0.82 $\pm$ 0.07   | 0.62 $\pm$ 0.08   | 0.23 $\pm$ 0.07   |
| Spleen      | 0.37 $\pm$ 0.09   | 0.37 $\pm$ 0.07   | 0.12 $\pm$ 0.06   |
| Kidney      | 8.30 $\pm$ 1.20   | 4.63 $\pm$ 1.01   | 4.32 $\pm$ 1.64   |
| Stomach     | 0.21 $\pm$ 0.04   | 0.17 $\pm$ 0.05   | 0.12 $\pm$ 0.01   |
| Intestine   | 0.44 $\pm$ 0.02   | 0.41 $\pm$ 0.19   | 0.20 $\pm$ 0.07   |
| Heart       | 0.17 $\pm$ 0.03   | 0.13 $\pm$ 0.01   | 0.08 $\pm$ 0.02   |
| Lung        | 0.42 $\pm$ 0.13   | 0.36 $\pm$ 0.25   | 0.36 $\pm$ 0.07   |
| Bone        | 1.15 $\pm$ 0.17   | 0.87 $\pm$ 0.07   | 0.77 $\pm$ 0.16   |
| Muscle      | 0.24 $\pm$ 0.02   | 0.232 $\pm$ 0.003 | 0.12 $\pm$ 0.03   |
| U87MG tumor | 6.54 $\pm$ 2.48   | 3.21 $\pm$ 0.38   | 1.93 $\pm$ 0.27   |
| 22Rv1 tumor | 4.30 $\pm$ 0.49   | 3.03 $\pm$ 1.18   | 1.60 $\pm$ 0.19   |

## 5. Radiopharmaceutical parameters

**Table S2.** Radiochemical yields, molar activities, and injected mass of [<sup>177</sup>Lu]Lu-PSFA-1 for in vivo studies.

| Experiment                       | RCY (%) | Molar activity (MBq/nmol) | Injected mass (µg/ mouse) |
|----------------------------------|---------|---------------------------|---------------------------|
| Micro-SPECT/CT Imaging           | 91.5    | 13.28                     | 0.78-0.80                 |
| Biodistribution study            | 94.8    | 12.87                     | 0.079-0.084               |
| Acute Toxicity Test              | 94.3    | 10.53                     | 9.94-10.17                |
| Radioligand Therapy Study(30MBq) | 95.1    | 10.46                     | 3.98-4.15                 |
| Radioligand Therapy Study(60MBq) | 95.1    | 10.46                     | 7.96-8.17                 |