Supplementary Methods for

First-in-human study of the radioligand ^{68}Ga-N188 targeting nectin-4 for PET/CT imaging of advanced urothelial carcinoma

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SUPPLEMENTARY MATERIALS AND METHODS

Chemical synthesis

General

All solvents and chemicals purchased from commercial sources were analytical grade or better and were used without further purification. The final products were characterized with high resolution mass spectrum (HRMS) using an AB SCIEX TripleTOF^{TM} 5600 (AB Sciex, Concord, Canada). High performance liquid chromatography (HPLC) analysis was performed using a Venusil MP C18 4.60 × 150mm^2 column (Bonna-Agela Technologies, Tianjin, China) on a FL-LC010G chromatography system (Bonna-Agela Technologies, Tianjin, China) with solvent gradient A (water with 0.1% trifluoroacetic acid) and gradient B (acetonitrile with 0.1% trifluoroacetic acid) at a flow rate of 0.8 mL/min. The analytical HPLC condition was gradient of 5−95% B over 5 min and 95% B for another 10 min (**method 1**), and the retention times were reported. Preparative HPLC purifications were performed using a Phenomenex C18 Luna 10.0 × 250 mm^2 column on a FL-H050G preparative chromatography system (Bonna-Agela Technologies, Tianjin, China). The products were eluted using eluent A (water with 0.1% trifluoroacetic acid) and eluent B (acetonitrile with 0.1% trifluoroacetic acid) at a flow rate of 4 mL/min (0-5 min, 5% B; 5-15 min, 5%-35% B; 15-25 min, 90% B; 25-35 min, 90% B) (**method 2**). The radiochemical purity (RCP) of ^{68}Ga-labeled compounds was analyzed on a radio-HPLC (20A, Shimadzu, Japan) with an analytical RP18 10 μm (4.6 mm × 250 mm) and the HPLC condition was gradient of 5−95% B (acetonitrile with 0.1% trifluoroacetic acid) over 5 min and 95% B for another 15 min (**method 3**).

Synthesis of nectin-4 targeting ligand N-188 and FITC-N188

The peptide resin (0.3 mmol/g) was purchased (A^+ Peptide, Shanghai, China) with all reactive side chain groups protected, as shown in Figure S1. The ligand was activated according to the following protocol. 200 mg resin was swelled in 2 mL dichloromethane (DCM) and washed with N,N-Dimethylformamide (DMF) for three times. The resin reacted with 2 mL DMF solution containing 20% piperidine three times (2 min, 10 min and 10 min) for deprotection, and then washed with 2 mL DMF twice (5 min).

For synthesis of **N188** (Figure S1), 2 equivalents DOTA-tris-tert-butyl ester (Tri-tert-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate, 68.8 mg, 0.12 mmol), activated by 2.4 equivalents HBTU (O-benzotriazole-tetramethylurea hexafluorophosphate, 54 mg, 0.144 mmol), HOBt (1-Hydroxybenzotriazole, 20 mg, 0.144mmol) and DIPEA (*N,N*-Diisopropylethylamine, 50 μL, 0.3 mmol) were added to the activated resin and reacted for 1 h at room temperature to generate intermediate **1**. The subsequent deprotection and cleavage were performed in 5 mL 95:2.5:2.5 (v/v/v) mixture of trifluoroacetic acid (TFA): triisopropylsilane (TIPS): water for 2 h. The filtrate was washed by 2 mL TFA. The solution was combined and distillated under reduced pressure to afford intermediate **2**. 50 mg intermediate **2** was dissolved in 10 mL pH = 8.0 buffer (20 mM NH_4HCO_3, 5 mM EDTA) with 20% acetonitrile and TATA (1,3,5-triacryloyl-1,3,5-triazinane, 12.4 mg, 0.06 mmol), and reacted at 30 °C for 60 min. The cyclization reaction was terminated by adding 10 equivalents cystine (72.6 mg, 0.6 mmol). **N188** was obtained by HPLC purification (**method 2**) and dried by a vacuum freeze dryer as a white solid (10.0 mg, 18%). HRMS calculated for $C_{113}H_{155}N_{27}O_{33}S_4$ $[M+2H]^{2+}$, 1273.0076, found 1273.0078 (Figure S3).

For synthesis of **FITC-N188** (Figure S2), 3 equivalents FITC (Fluorescein 5-Isothiocyanate, 70.1 mg, 0.18 mmol), and DIPEA (*N,N*-Diisopropylethylamine, 100 μL, 0.6 mmol) were added to the activated resin and reacted for 2 h at room temperature to generate intermediate **3**. The subsequent deprotection and cleavage were performed in 5 mL 95:2.5:2.5 (v/v/v) mixture of trifluoroacetic acid (TFA): triisopropylsilane (TIPS): water for 2 h. The filtrate was washed by 2 mL TFA. The solution was combined and distillated under reduced pressure to afford intermediate **4**. 40 mg intermediate **4** was dissolved in 10 mL pH = 8.0 buffer (20 mM NH_4HCO_3, 5 mM EDTA) with 20% acetonitrile and TATA (1,3,5-triacryloyl-1,3,5-triazinane, 11.1 mg, 0.052 mmol), and reacted at 30 °C for 60 min. The cyclization reaction was terminated by adding 10 equivalents cystine (63.1mg, 0.52 mmol). **FITC-N188** was obtained by HPLC purification (**method 2**) and dried by a vacuum freeze dryer as a yellow solid (2.0 mg, 4.5%). HRMS calcd for $C_{118}H_{138}N_{24}O_{31}S_5$ $[M+2H]^{2+}$, 1274.4354, found 1274.4333 (Figure S4).

Affinity determination

To determine **N188** affinity to nectin-4, we used surface plasmon resonance technology (SPR), the experiments were conducted at Shanghai Medicilon Inc. using a SPR biosensor (Biacore 8K/2234033, USA) to get the K_d value. Data was collected under the condition of pH = 4.0, 10 μ/mL Nectin-4, 1:1 binding.

Radiolabeling

^{68}[Ga]$GaCl_3$ was obtained from a ^{68}Ge/ ^{68}Ga generator (maximum production 1.85 GBq, ITG, Germany). ^{68}Ga-labeling was performed by heating 3 mL 0.05 M HCl solution containing ^{68}[Ga]$GaCl_3$ (740-1110 GBq), 200 μL of 1.0 M sodium acetate and 100 μg **N188** at 90 °C for 10 minutes. After cooling down, solution was extracted by an activated C18 column (the C18 column was activated by 10 mL ethanol and 10 mL water) and the radiolabeled ligand was eluted by 1 mL 80% ethanol aqueous solution. After purification, the radiolabeled ligand was obtained with >99% (n = 19) radiochemical purity analyzed by radio-HPLC. 592-888 GBq could be obtained with the radiochemical yield of ~50.3% (non-decay corrected, n = 19).

Stability and solubility studies

The partition coefficients (Log *P*) were determined in phosphate-buffered saline (0.1 M, pH=7.4)/1-octanol (v/v = 1:1). In a 15-mL centrifuge tube, 0.1 mL of ^{68}Ga-labeled compound (37–74 KBq), 1.9 mL PBS, and 2.0 mL 1-octanol were mixed. The mixture was vortexed for 1 min and then centrifuged at 5000 rpm for 3 min. Three samples (100 μL) from each layer were measured using a γ-counter (Hidex AMG, Sheffield, United Kingdom). The experiment was performed in triplicate. The partition coefficient was calculated as the average counts in 1-octanol divided by the average counts in PBS, and the value was expressed as Log *P* ± SD. To evaluate the stabilities of radioligands *in vitro*, the ^{68}Ga-labeled compound was incubated in saline at room temperature or in mouse serum at 37 °C for 0.5 h, 1h and 2h. After the addition of 1 mL acetonitrile, the mixture was homogenized for 5 min and centrifuged for another 5 min. Supernatant was then analyzed by radio-thin-layer chromatography (iTLC).

Pharmacokinetics

Female Kunming mice (n = 4) were injected with ^{68}Ga-labeled ligands (7.4 MBq, 100 μL) *via* the tail vein. 100 μL of blood was taken from the mice orbit vein at different times after the injection (10s, 30s, 60s, 120s, 300s, 600s, 1800s, 3600s, 7200s). The quantification of blood radioactivity was performed on a γ-counter (Hidex AMG, Sheffield, United Kingdom). After calculating the %ID/g at different time points, fast half-life and slow half-life were given using a two-phage decay model in Prism (San Diego , California, USA).

Toxicity test

In order to verify the toxicity of **^{68}Ga-N188**, 6 Kunming mice (18-22 g) were used in the test with 3 mice as the control group, and the weights of the mice were recorded before the test. The experimental group was injected with 18.5 MBq/0.1 ml **^{68}Ga-N188** solution (925Bq/kg, 750 times the dose administered to humans) through the tail vein of mice, and the control group was injected with the same volume of saline. The mice were monitored for 14 days including diet, respiration, activity, defecation, skin and pain sensation and other indicators. During the observation period, all the animals were alive and have no abnormal reactions. After observation, all mice were sacrificed. Main organs-heart, lung, liver, spleen and kidney, were fixed with paraformaldehyde, embedded with paraffin, sliced and conducted HE staining. The results warrant the safety to apply **^{68}Ga-N188** for clinical study.