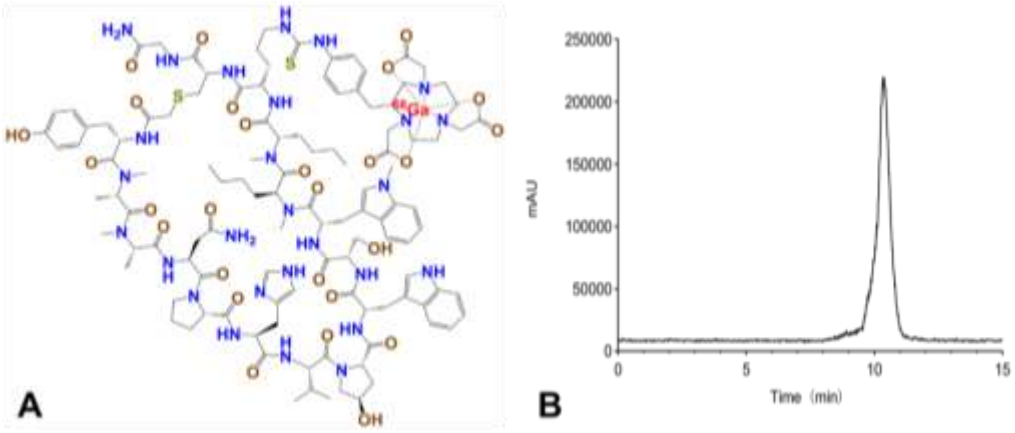
Radiolabeling and Purification of ^{68}Ga-NOTA-WL12

NOTA-WL12 was obtained from Chinapeptides Company (Shanghai, China) as a custom service standard. ^{68}Ga was obtained from a ^{68}Ge/^{68}Ga radionuclide generator (ITG, Germany) and used to label the NOTA ligand. ^{68}Ga-NOTA-WL12 was synthesized by incubating $^{68}GaCl_3$ with the ligand in pH = 4.0 buffer at 60°C for 15 min. After Sep-Pak C18 (Waters, Germany) cartridge purification, the radiotracer was obtained by radio high-performance liquid chromatography (HPLC), and the radiochemical purification yield was 99% (Supplemental Figure 1). As shown in Supplemental Table 1, the ^{68}Ga-NOTA-WL12 solution complied with the Regulation of the Administration and Preparation of Positron Radiation Drugs by Medical Institutions. Radiopharmaceuticals are subjected to quality control analysis mainly to ensure their safety. The radiolabeled production of ^{68}Ga-NOTA-WL12 was typically 555-740 MBq with 30 μg of NOTA-WL12. The injection dose, ranging from 1.9-3.7 MBq/kg, was decided based on the patient's weight. Thus, the masses of the injected radiolabeled compounds injected differed, ranging from approximately 10-20 μg.



Supplemental Figure 1. Structure of ^{68}Ga-NOTA-WL12 (A). HPLC analysis of ^{68}Ga-NOTA-WL12 (B).

Supplemental Table 1. The quality control results of ^{68}Ga-NOTA-WL12

Parameter	QC Specification	QC Result
Appearance	Clear,colorless	pass
Volume	2.0-5.0mL	4.0 mL
pH	5.0-8.0	7.4
Radio-TLC	>95%	>99%
Radio-HPLC	>95%	>99%
Ethanol	<5%	<5%
Endotoxins	<15EU/mL	< 5EU/mL
Sterility	Sterile	Pass
Specific Activity	18.5- 296 GBq/μmol	74 GBq/μmol

The radiochemical purity was analyzed by radio HPLC (Supplemental Figure 1B), which was conducted on a C18 column (Zorbax 300SB-C18, 4.6×250 mm, 5 μm; Agilent, USA) with (A) H_2O (0.1% TFA) and (B) acetonitrile (0.1% TFA) using a linear A-B gradient from 95/5 (A/B) to 20/80 (A/B) over 15 min period with a flow rate of 1 mL/min and a UV wavelength of 220 nm. Radio TLC was conducted using iTLC-SG-Glass microfiber chromatography paper containing silica gel (Agilent Technologies, USA) on an AR 2000 system (Bioscan, USA). The pH of the injectate was measured by a pH meter (SP-2500, SUNTEX, China). The limulus test was performed to assay endotoxins. A direct inoculation method was employed to assess the sterility of the ^{68}Ga-NOTA-WL12 solution.

The in vitro stability test of ^{68}Ga-NOTA-WL12 was performed in saline and in 5% human serum albumin (HSA) at 37 ℃ at 30, 60 120 and 240 min. At different time points, 20 μL of the mixture was removed and used for the analysis of radiochemical purity by radio TLC (n=3). The radiochemical purity of the radiotracer in both solutions was over 95% after 240 min of incubation.

Radiotoxicity in mice

In the radiotoxicity study, fourteen BALB/c mice (16~19 g, female, 4~6 weeks old) were randomly divided into two groups. In the experimental group, each mouse was injected with 0.5 mL of ^{68}Ga-NOTA-WL12 at a dose of 1.57 GBq/kg (over 500-fold more than the dose used in human subjects). In the control group, each mouse was injected with 0.5 mL of saline. At 1, 2, 3,

4, 5, 6 and 7 days postinjection, the mice were weighed, and routine blood tests were performed by the Animal Laboratory of Peking University Health Science Center.

Cell culture , flow cytometric analysis and cell uptake study.

CHO and CHO-hPD-L1 cells were cultured in F-12K medium supplemented with 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO_2. The CHO and CHO-hPD-L1 cell lines were subjected to flow cytometric analysis. Single CHO and CHO-hPD-L1 cell suspensions were stained with PE-conjugated anti-human CD274 (PDL1) (329705, BioLegend) at a final concentration of 1 μg/ml for 30 min at 4°C in the dark. Subsequently, dead cells were eliminated by adding 1 μg/ml propidium iodide (PI) for 10 min at room temperature. Flow cytometry analyses were performed on a BD FACSCalibur™ instrument (BD Biosciences). Data were analyzed with Flow Jo software (version 10.6.2).

CHO-hPD-l1 and CHO cells were plated on 24-well cell plates (1×10^6 cells per well) 24 hours before the study. Half a milliliter of fresh medium containing 1 μCi of the radiotracer was added to each well, and the cells were cultured in an incubator at 37 ℃ with 5% CO_2. After incubation for 5, 30, 60 and 120 min, the medium was removed, and cells in each well were then washed 2 times with 1 mL of cold phosphate-buffered saline (PBS) and lysed with cold NaOH (1 *M*). In the blocking study, cells in each well were co-incubated with 20 μg of the WL12 peptide. The radioactivity of cells in each well was measured with a γ-counter, and the results are shown as the percentage of the added dose (% AD) / 10^6 cells.

Flow cytometric analysis and small-animal PET imaging

Small-animal PET Imaging

NOD/SCID mice bearing CHO-hPD-L1 tumors and CHO tumors were intravenously injected with ~7.4 MBq (~0.17μg) of ^{68}Ga-NOTA-WL12 in 200 μL of saline. The mice were anesthetized with 3% isoflurane before the micro-PET scan, and 1.5% isoflurane was maintained during the scanning. Fifteen-minute static micro-PET imaging was performed at 0.5, 1 and 2 h after injection. In the blocking study, the mice were coinjected with 50 μg of unlabeled WL12. Fifteen-minute static micro-PET scans were then acquired at 1 and 2 h after injection. Imaging was performed on a Super Nova PET scanner using an 80-mm-diameter transaxial field of view, and ordered-subset expectation maximization 3-dimensional reconstruction algorithms with attenuation and random corrections were performed. The final images were displayed by Avatar3.lnk workstation software. In the PET/CT imaging analysis, the region of standardized uptake value (SUV) was calculated. To further confirm the PD-L1 expression level in tumor cells, PD-L1 immunohistochemistry was conducted on CHO-hPD-L1 tumors and CHO tumors.

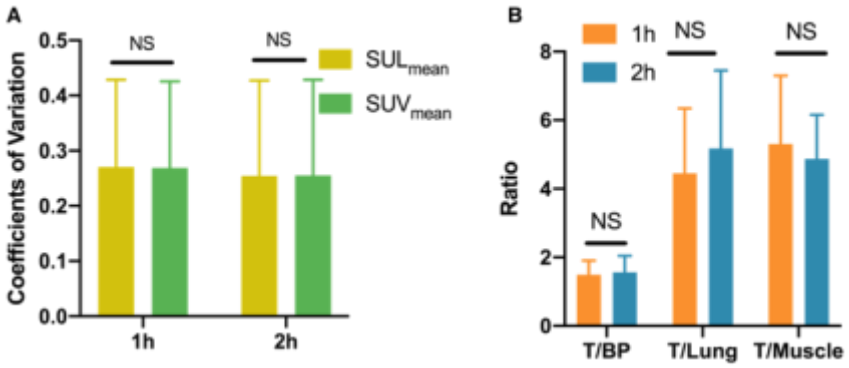
Radiation Dosimetry Calculations.

Methods for dosimetry analysis.

The heart, lung, liver, spleen, pancreas, kidneys, uterus, urinary bladder and remainder of the body were selected as source organs. The volume of organs was determined manually by making an outline of the organ on CT imaging layer by layer. Whole-organ activity (MBq) was calculated by multiplying organ volume by mean counts/mL and dividing by 1000. The percentage of injected activity was calculated to generate the activity concentration-time curve of each source organ with no consideration of the biological half-life. Time-integrated activity coefficients were calculated individually, biexponential curve fitting was applied to activity concentration-time curves of the uterus and urinary bladder content, while mono-exponential curve fitting was applied to other source organs, to calculate the areas under the curve, and human organ dosimetry was further estimated using OLINDA/EXM software (version 2.0; Hermes Medical Solutions AB). Organ-absorbed doses and total effective doses, as well as residence time for each patient, were obtained using reference adult male and female models.

Supplemental Imaging analysis

The comparison of coefficients of variation between SUV/L_{mean} and ratio of tumor to background at 1h and 2h (Supplemental Figure 2).



Supplemental Figure. 2 The coefficient of variations of organs in 1 and 2 h made no differences between SUL_{mean} and SUV_{mean} (A). The ratios of tumor to background (blood pool, lungs, and muscle), as T/BP, T/Lung and T/Muscle, were not significantly different at 1 h and 2 h (B). NS, no significant differences.

Supplemental Imaging analysis.

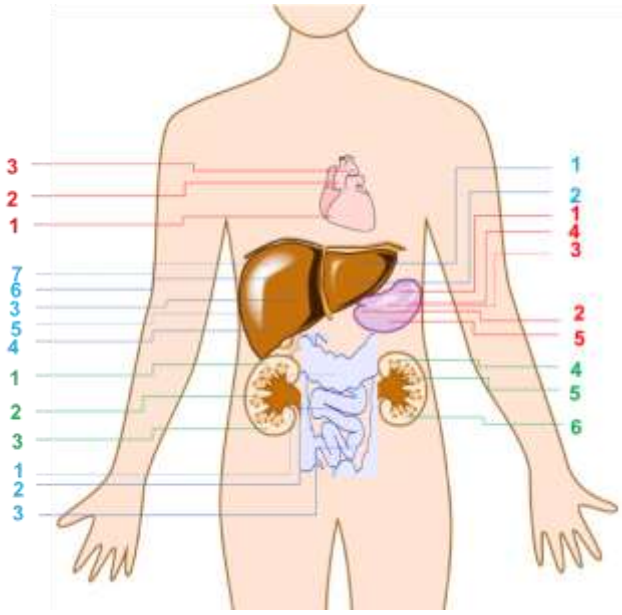
Supplemental Table. 2 Comparison of Coefficients of Variation between SULmean and SUVmean at 1 and 2 h after injection

	1 h		2 h	
	SUL_{mean}	SUV_{mean}	SUL_{mean}	SUV_{mean}
Heart	0.15772569	0.16017626	0.1283289	0.12699374
Blood Pool	0.24965731	0.24769874	0.28701308	0.29322299
Lung	0.28079196	0.27352511	0.22403846	0.2322501
Liver	0.19453637	0.18744827	0.08292365	0.08019268
Spleen	0.18443101	0.18718625	0.1596635	0.16388395
Muscle	0.134009	0.13312701	0.16478173	0.16248077
Bone Marrow	0.22410873	0.22777378	0.330907	0.32710491
Small Intestine	0.63201945	0.62745836	0.62348588	0.62479503
Tumor	0.32675577	0.32793333	0.31942288	0.32345226

Supplemental Imaging analysis.

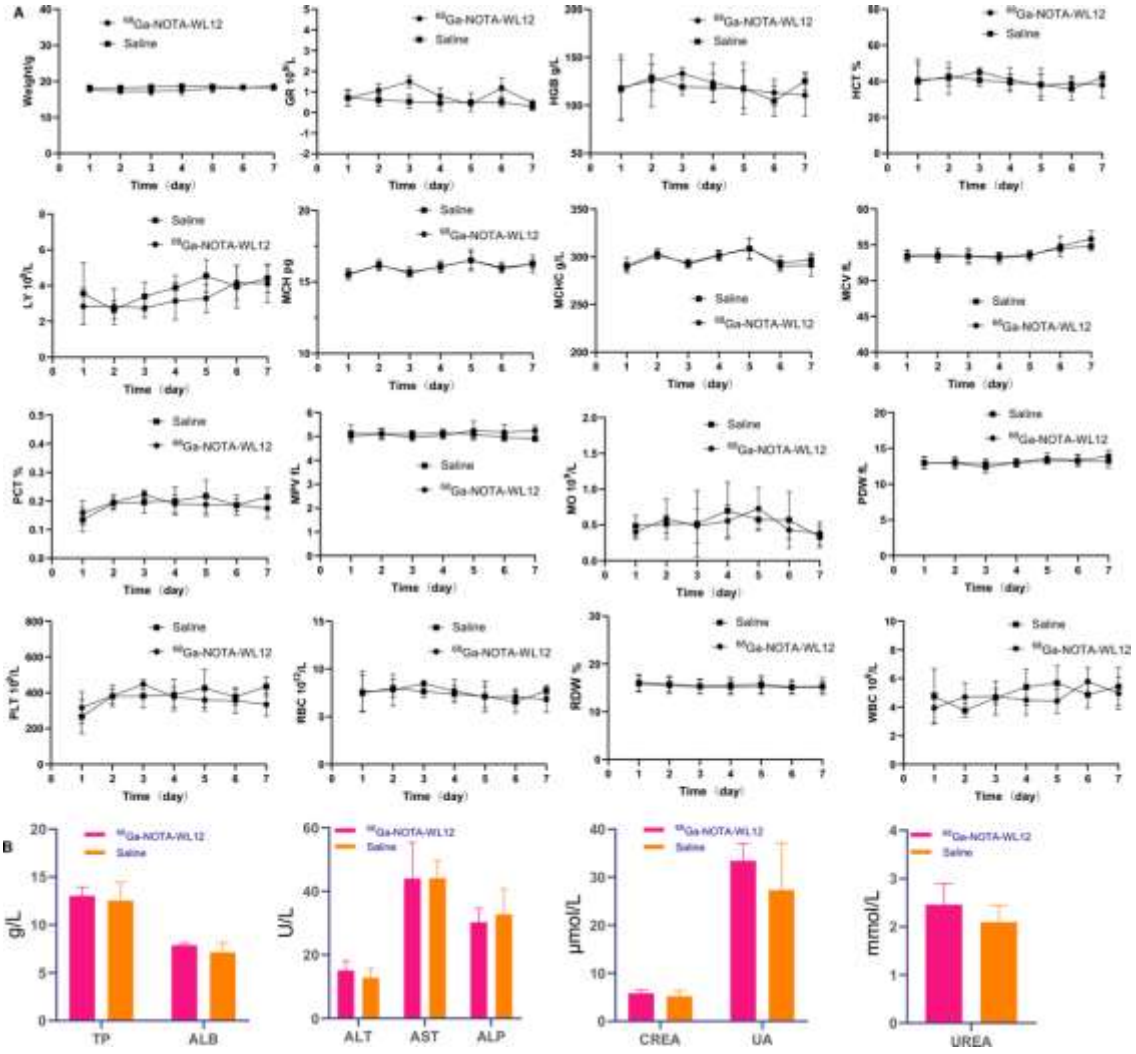
Uptake measurements of normal organs (Supplemental Figure 3).

The uptake of some normal organs was measured by multi-sites to make a more precise result for comparison. The blood pool was measured in the right ventricle, ascending aorta and descending aorta(totally 3 sites). The liver was measured in segments II-VIII (7 sites in total). The spleen was measured at superior, inferior, left, right and central positions (a total of 5 sites). Each kidney was measured at superior, central and inferior positions (a total of 6 sites). And the Small intestine was measured in upper, middle and lower abdomen(totally 3 sites). And the specific sites of the organs were depicted as follows.



Supplemental Figure 3. Uptake measurements of normal organs

Radiotoxicity in mice

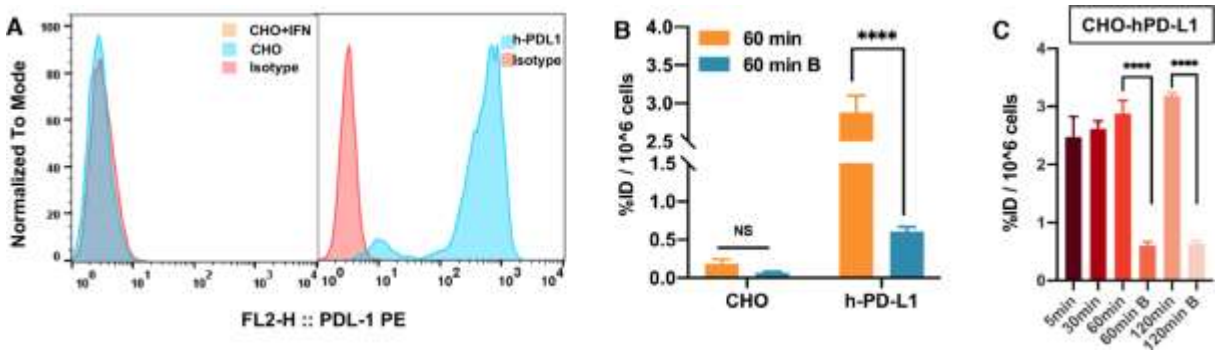


Supplemental Figure 4 (A): The results of weight and routine blood test of mice in radiotoxicity study of ^{68}Ga-NOTA-WL12 (n=7). GR (neutrophil), HGB(hemoglobin), HCT (hematocrit) , LY (absolute value of lymphocytes), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), MCV (mean corpuscular volume) , PCT (platelet counts), MPV (mean platelet volume), MO (absolute value of monocyte), PDW (platelet distribution width), PLT (platelet counts), RBC (red blood cell count), RDW(red blood cell distribution width), WBC (white blood count). (B): The results of biochemical tests. TP (total protein), ALB (albumin), ALT

(alanine aminotransferase), AST (aspartate amino transferase), ALP (alkaline phosphatase), CREA (creatinine)，UA (Uric Acid), UREA (carbamide).

In Vitro Cellular Studies and Small-Animal PET Imaging.

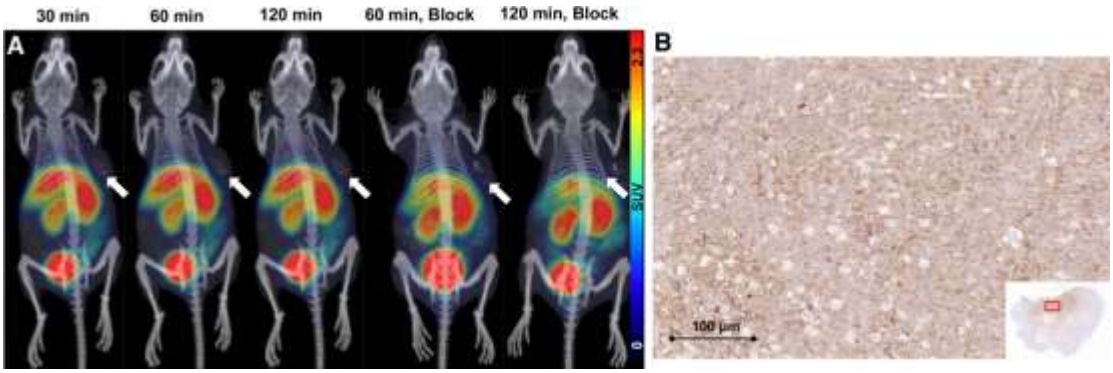
Uptake of ^{68}Ga-NOTA-WL12 of different cell lines in vitro and in vivo (Supplemental Figure 5).



Supplemental Figure. 5 Flow cytometric analysis of CHO and CHO-hPD-L1. (A). In vitro uptake of ^{68}Ga-NOTA-WL12 of CHO and CHO-hPD-L1 at 60 and 120 min (B). Cell uptake of ^{68}Ga-NOTA-WL12 without and with a blocking dose in CHO-hPD-L1 cell lines (C).

In Vitro Cellular Studies and Small-Animal PET Imaging.

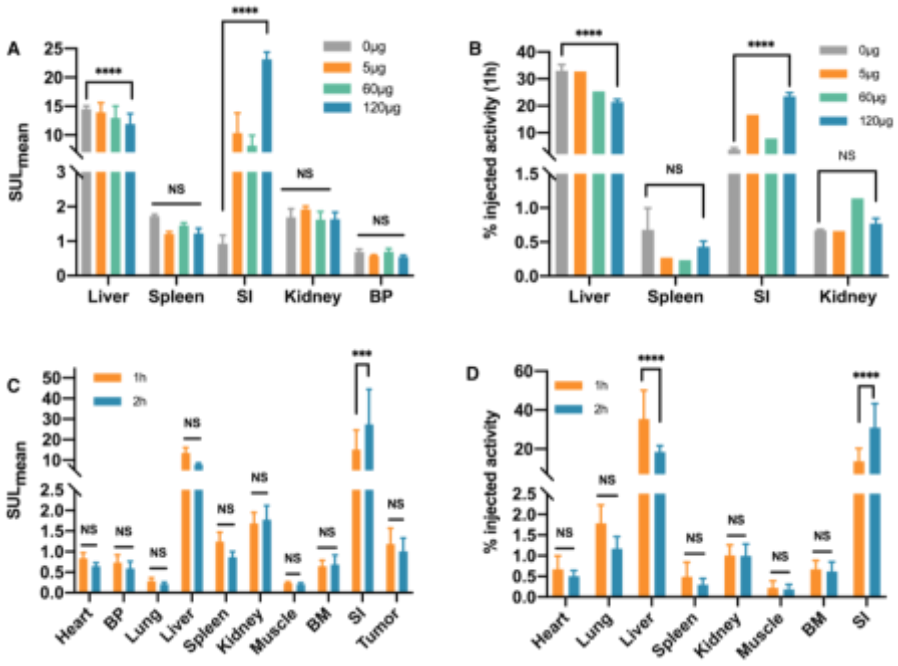
^{68}Ga-NOTA-WL12 uptake in negative control CHO tumor model by small-animal PET imaging (Supplemental Figure 6A). Immunohistochemistry analysis of PD-L1 expression in CHO-hPD-L1 tumors(Supplemental Figure 6B).



Supplemental Figure. 6 A, ^{68}Ga-NOTA-WL12 PET/CT imaging without and with blocking dose in NOD/SCID mice bearing CHO tumors at different time points. B, Immunohistochemistry analysis of PD-L1 expression in CHO-hPD-L1 tumors (100 μm).

Biodistribution in NSCLC patients.

The comparison of radioactivity and percentage of injection (Supplemental Figure 7)



Supplemental Figure 7. SUL_{mean} of liver, spleen, small intestine (SI), kidney and blood pool (BP) given different doses of WL12(0, 5, 60 and 120 μg) at 1h after injection (A). Percentage of injection of high-uptake normal organs given different doses of WL12 (B). Comparison of SUL_{mean} of normal organs and tumors at 1and 2 h (C). Comparison for percentage of injection of normal organs at 1and 2 h (D).

Tumor uptake and correlation with immunohistochemistry

The relationship between tumor uptake(^{68}Ga-NOTA-WL12 and ^{18}F-FDG) and TPS of PD-L1 immunohistochemistry (IHC) expression (Supplemental Table 2).

Supplemental Table 3. Relationship of tumor radioactivity uptake with PD-L1 IHC.

	^{68}Ga-NOTA-WL12		^{18}F-FDG	
	R_S *	*P**	R_S	*P*
SUV_{max}	0.86	0.0029	0.576	0.1046
SUL_{max}	0.7643	0.0165	0.4541	0.2195
SUV_{peak}	0.9349	0.0002	0.5529	0.1226
SUL_{peak}	0.8469	0.004	0.4261	0.2528
SUV_{mean}	0.867	0.0025	0.6937	0.0382
SUL_{mean}	0.7723	0.0147	0.5864	0.097
T/BP (SUV)#	0.5313	0.141	0.3491	0.3571
T/BP (SUL)#	0.7479	0.0205	0.3384	0.3731

*: R_S, square of correlation coefficient from Spearman correlation; *P*: value for significance; #: ratio of tumor SUV/L_{max} to blood-pool SUV/L_{mean}. IHC, immunohistochemistry.

Relationship of ^{68}Ga-NOTA-WL12 uptake and therapy evaluation.

Tumor radio-uptake and therapy evaluation (Supplemental Table 4).

Supplemental Table 4. Tumor radioactivity uptake and therapy evaluation

Patient No.	PD-L1 TPS	$SUV_{max,}$ WL12	$SUV_{max,}$ FDG		PERCIST	RECIST
		Before Therapy	Before Therapy	After Therapy		
1	8%	2.21	9.13	3.54	PMR	SD
3	8%	1.84	16.55	21.38	PD	PD
6	30%	3.05	8.03	3.1	PMR	SD

Partial metabolic response (PMR); Stable disease (SD); Progressive disease (PD).

1. Chatterjee S, Lesniak WG, Gabrielson M, et al. A humanized antibody for imaging immune checkpoint ligand PD-L1 expression in tumors. *Oncotarget.* 2016;7:10215-10227.