Supplemental Data

Materials

All reagents were obtained from Sigma-Aldrich. CLDN18.2-targeted nanobody (ACN376) was selected by Chengdu AlpVHHs Co.ltd. The LPETG-His_6 modified CLDN18.2-targeted nanobody (ACN376) was provided by Prof. Bing Jia (Medical Isotopes Research Center and Department of Radiation Medicine, Beijing China). GGGGC oligopeptide was custom synthesized by GL Biochem (Shanghai, China). Maleimido-mono-amide-NOTA TFA Salt was purchased from Toronto Research Chemicals (Toronto, Canada). ^{68}Ga was obtained from a ^{68}Ge/^{68}Ga generator produced by ITG (Isotope Technologies Garching GmbH) in the Department of Nuclear Medicine, Peking University Cancer Hospital. Disposable PD-10 Desalting Columns (PD-10 columns) were purchased from GE Healthcare (Piscataway, NJ, USA). The sterile filter (0.22 μm) was purchased from PALL (New York, USA).

Synthesis and characteristic of ^{68}Ga-NC-BCH

The ACN376 was modified with LPETG-His_6 at its C-terminus, which could be recognized by sortase A. Then the radiolabeling precursor of NOTA-ACN376 was prepared by sortase A-mediated transacylation. Briefly, ACN376 (0.4 mM) was mixed with sortase A (8 μM) and GGGGC oligopeptide (10 mM) in reaction buffer (0.1 M Tris-HCl, pH 7.5, 2 M NaOH, 1 M $CaCl_2$) for 3 h at 4 ℃ to get ACN376-G_4C. After purification by size-exclusion chromatography in 0.01 M PBS solution, ACN376-G4C was mixed with five molars excess Mal-NOTA to incubate for 1 h at 30 °C under basic conditions (pH = 7.0). NOTA-ACN376 was isolated from the reaction mixture by size-exclusion chromatography in 0.01 M PBS solution. The mass spectra of ACN376 and NOTA-ACN376 were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Sciex, USA).

The radionuclide ^{68}Ga was eluted from a ^{68}Ga/^{68}Ge generator using 0.05 M HCl (4 mL) as

the fractionated eluent. Then NOTA-ACN376 (200 μg) was mixed with metallic cation $^{68}Ga^{+}$ (1 mL) and sodium acetate (1 M, 65 μL). The reaction mixture was incubated at 37 °C for 15 min and purified by a PD-10 column with 0.01 M PBS as the eluent. The radiochemical yield and radiochemical purity of 68Ga-NC-BCH were measured by radio-thin-layer chromatography (Radio-TLC) in a standard protocol.

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 68Ga-NC-BCH (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. The vitro stability was performed that ^{68}Ga-NC-BCH incubated in 5% human serum albumin (HSA) and 0.01 M PBS, and the radiochemical purity was analyzed by Radio-TLC at 0.5, 2, 4 and 6 h.

Cell and animal models

The cells were cultured in RPMI-1640 medium which was supplemented with 10% FBS plus 1% antibiotics from Invitrogen. For PET/CT imaging and vitro biodistribution experiments, AGS and AGS$^{CLDN18.2}$ xenografts were established in 4~6-week-old female BALB/c nu/nu mice which were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The right axillary of the mouse was subcutaneously injected with 5×10^6 AGS/AGS$^{CLDN18.2}$ cells suspended in 100 μL PBS. Tumors were grown for 5~6 weeks to reach an average volume of 100 mm^3. Mice were raised under specific disease-free conditions and were handled and maintained according to the Institutional Animal Care and Use Committee guidelines.

Small-animal PET/CT protocol

^{68}Ga-NC-BCH (7.4 MBq in 200 μL solution) was injected intravenously to each animal via tail vein for PET studies. The AGS$^{CLDN18.2}$ tumor-bearing mice were chosen for blocking experiments, and each animal was injected with 1 mg TST001 antibody 24 h in advance. The regions of interest (ROI) were used to estimate the uptake in each organ. With a small-animal PET/CT scanner (Super Nova PET/CT, Pingseng Healthcare, Shanghai, China), the PET images were reconstructed by Avatar 3, and the ROI-derived standard uptake value (SUV) was calculated by drawing ROIs over these organs.

Binding of ^{68}Ga-NC-BCH to CLDN18.2

ELISAs were used to determine the binding potency between the ^{68}Ga-NC-BCH and CLDN18.2 protein. First, 100 μL solution of the human CLDN18.2 full length protein-VLP (CL2-H52P7, 2 μg/mL) was added to each well coated with a 96-well polystyrene StripwellTM microplate (Corning Costar, CLS2481-100EA), at 4 °C overnight. Then the antigen solution was discarded, and washed five times with PBST. After that, 5% powdered milk (diluted with PBS) was added to the microplate for 2 h at 37 °C to block other nonspecific sites. Then, after discarding the 5% powdered milk, five times washed with PBST. When the CLDN18.2 protein plates were ready, ^{68}Ga-NC-BCH was added with thirteen concentration gradients, 0.003, 0.015, 0.03, 0.15, 0.3, 3, 6, 15, 30, 60, 150, 300 and 1110 kBq, 100 μL per well (n = 4), then incubated for 1 h at 37 °C. Then, the solution was discarded, and the samples were washed five times with PBST. Finally, the radioactivity of each incubation well was analyzed by a fully automatic γ-counter (Perkin Elmer, Wizard2).

Cellular uptake was studied according to a previous report (10). AGS / AGS$^{CLDN18.2}$ cells were cultured in RPMI-1640 culture medium (2.0×10^5 cells/mL) and added to a 24-well plate (1.0 mL per well) to culture overnight. ^{68}Ga-NC-BCH (20 μL, 37 kBq, 6.87×10^{-12} M) was added to wells (n = 4) containing adherent AGS$^{CLDN18.2}$ / AGS cells. The mixture was incubated in a 5% CO_2

incubator at 37 °C for 2, 10, 30, 60, and 120 min. After incubation, the culture medium was removed and the cells were washed 2 times with cold PBS (0.01 M). For a competitive binding assay, both two tumor cells uptake of ^{68}Ga-NC-BCH was studied with and without pretreatment with a 1,000-fold molar excess of unlabeled ACN376 or TST001. The CLDN18.2 antibody TST001 has been approved as an investigational new drug for the treatment of advanced or metastatic solid tumors (NCT02827968, NCT03101488, and NCT03248843). Blocking for 60 and 120 min was performed in the presence of excess unlabeled TST001 (50 μg). Then, the cells were collected after digestion by 1 M NaOH and counted in a gamma counter. The percentage of added dose per 2.0×10^5 cells (%AD/2.0×10^5 cells) was calculated according to the count.

Biodistribution study

The KM mice were intravenously injected with 0.74 MBq of ^{68}Ga-NC-BCH via the tail vein and were then sacrificed at 0.5, 1, 2 and 4 h p.i. (n = 4). The tissues including the blood, heart, liver, spleen, lung, kidneys, stomach, intestines, muscle, bone and brain were dissected. The radioactivity of the tissues was measured using a γ-counter. The radioactivity of each organ was calculated as % injected dose per gram (%ID/g). For the tumor model's ex vivo biodistribution, female nude mice bearing AGS$^{CLDN18.2}$ tumor xenografts were injected by tail vein with 0.74 MBq of ^{68}Ga-NC-BCH to evaluate the distribution of ^{68}Ga-NC-BCH in major organs and tumors (n = 4 per group). The mice were sacrificed and dissected at 48 h p.i. (n = 4), and the tumor, kidney, blood, and other major organs were collected and weighed. The blocking study was also performed in AGS$^{CLDN18.2}$ mice by an injection of excess dose of cold TST001 (1 mg) 24 h in advance. At 2 h p.i., the blocked mice were sacrificed and dissected. Then, the organ biodistribution of ^{68}Ga-NC-BCH was determined.

Pharmacokinetics

Female Kunming mice (n = 5) were injected with ^{68}Ga-NC-BCH (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-NC-BCH, 7 KM 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 37 MBq/0.1 ml 68Ga-NC-BCH solution (1233MBq/kg, 330 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-NC-BCH for clinical study.

Study design

The adult patients included in this study were histologically diagnosed with advanced gastric cancer or colorectal cancer, and their tumor tissues were confirmed to express CLDN18.2 by IHC. CLDN18.2 positivity was defined as moderate-to-strong CLDN18.2 expression $\geqslant$ 40% of tumor cells(*1*). The eastern cooperative tumor group (ECOG) had a physical state score of 0 or 1. Other eligibility criteria include: age 18 years or older; Life expectancy is at least 3 months; The exclusion criteria included any of the following: liver and/or renal dysfunction, pregnancy or current lactation, and inability to assume a supine position continuously on the scanner bed.

^{68}Ga-NC-BCH PET/CT scanning

An optimized dynamic whole body acquisition duration of 60 min. Subsequently, a whole-body static PET/CT scan was performed in 120 min that continued for 5 min. The remaining seven participants underwent a static total-body PET/CT scan at 60 min and 120 min after the injection respectively, which lasted 5 min.

^{18}F-FDG PET/CT scanning

Within one week after ^{68}Ga-NC-BCH PET/CT, all patients underwent a whole-body static ^{18}F-FDG PET scan within 60 minutes at an injection dose of 3.7 Mbq/kg. Prior to FDG injection, all patients fasted for at least 6 h. All subjects had blood glucose <200 mg/dL before injection. ^{18}F-FDG PET/CT collection conditions are the same as ^{68}Ga-NC-BCH PET/CT.

The dynamic ^{68}Ga-NC-BCH PET/CT imaging reconstruction

The dynamic PET images were reconstructed using the order subset expectation maximization algorithm. The 60 min scan was reconstructed into 100 frames: 15 frames of 2 s duration, 30 frames of 5 s, 30 frames of 14 s, and 25 frames of 120 s. The images were reconstructed using 3 iteration and 20 subsets, with time-of-flight and point spread function enabled. The matrix was 192-by-192, and the slice spacing was 2.886 mm. A 4.5 mm Gaussian filter was applied to the images.

Image Analysis

The dosimetry of ^{68}Ga-NC-BCH was studied using OLINDA/EXM software (version 2.0; Hermes Medical Solutions AB). A workstation (uWS-MI, United Imaging Healthcare) was used for post processing. Two experienced nuclear medicine/radiologists reviewed and analyzed the rests of ^{68}Ga-NC-BCH PET/CT and ^{18}F-FDG PET/CT independently, and any inconsistencies were resolved by consensus.

For normal organ biological distribution measurements, a spherical VOIs is mapped with a fixed size for each organ. Normal organs/tissues selected for ROI analysis include: brain, breast, esophagus, eyes, gallbladder, left colon, small intestine, stomach wall, right colon, rectum, kidneys, aorta, liver, spleen, pancreas, uterus, ovaries/prostate, thyroid, salivary glands and lungs. The weight corrected SUV was calculated by weight, injection activity, and the amount of radioactivity detected within the VOI. A positive lesion is defined as a tumor with a higher uptake of ^{68}Ga-NC-BCH than the local level of surrounding tissue. SUVmax of each positive lesion was measured separately. SUVmax values of 68Ga-NC-BCH PET and ^{18}F-FDG PET at 2 hours were analyzed. For lesion evaluation, we also calculated the T/NT ratio, that is, the ratio of SUVmax in the metastatic lesion to SUVmax in the liver background.

Statistical analyses

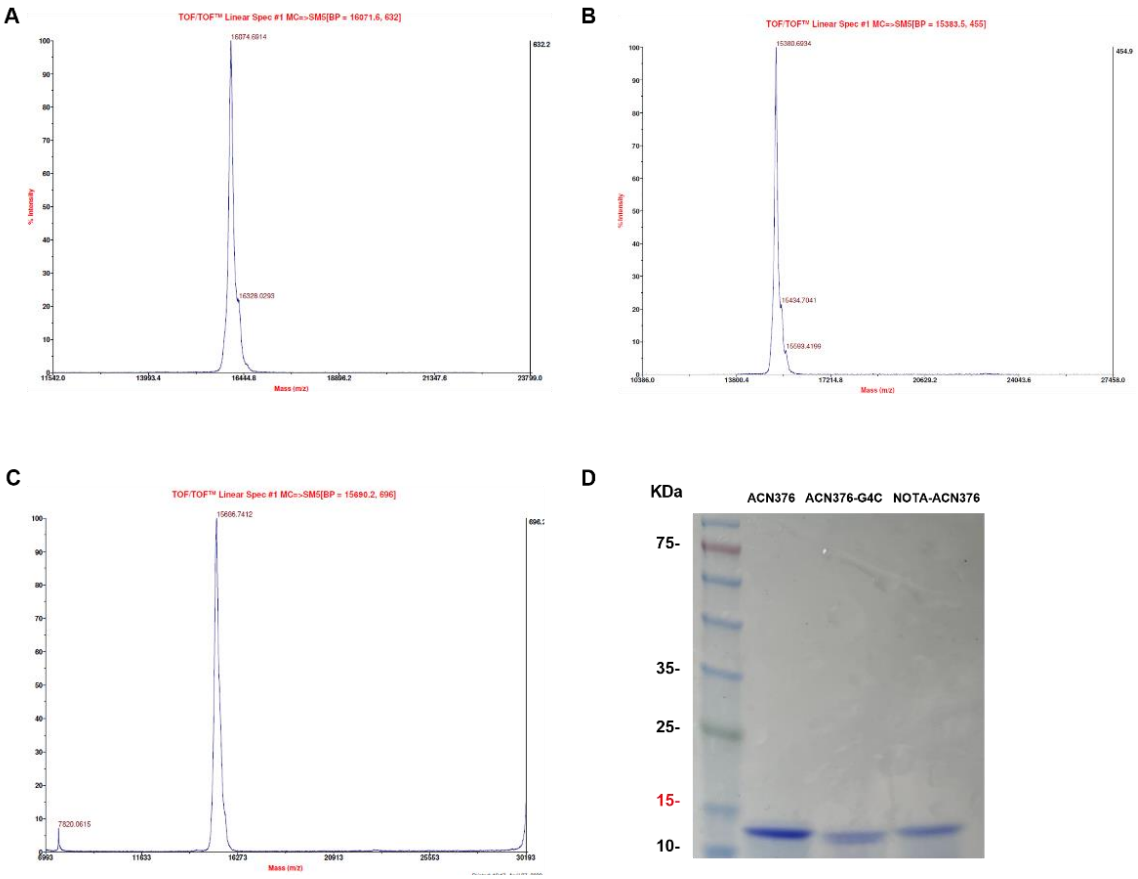
Statistical analyses were performed with Origin software (V2018, Microcal, USA) and Prism (V8.0, GraphPad Software, New Zealand). The cellular uptake, the blood biochemical parameters, biodistributions of tumor xenografts and other compared data between two independent samples were analyzed by unpaired Student's t tests. P-value lower than 0.05 was considered to be significant.

The organ uptake data in the form of maximum standard uptake value (SUVmax) were grouped by gender and age. To compare distributions among samples, the parametric continuous variables were expressed as mean ± SD. Independent sample t-tests were used to compare mean standard uptake value (SUVmean) or SUVmax or target-to-nontarget (T/NT) ratio values between different groups. P-value less than 0.05 was considered to be significant.

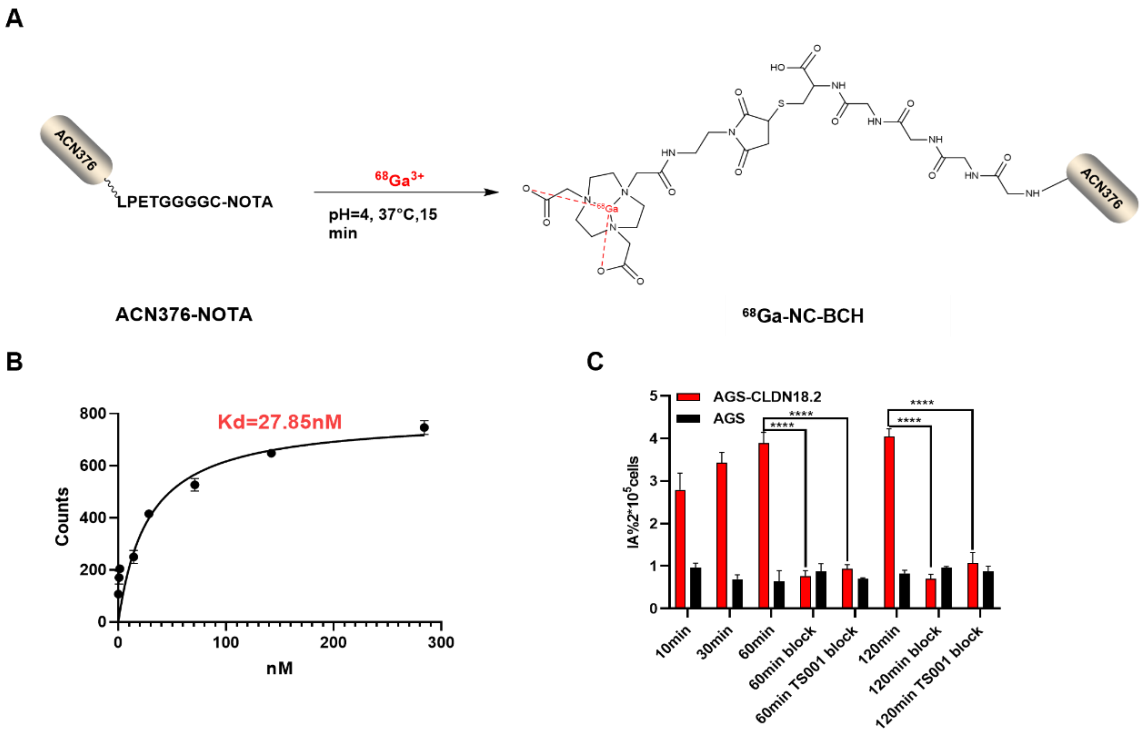
REFERENCE

1. Jia K, Chen Y, Sun Y, et al. Multiplex immunohistochemistry defines the tumor immune microenvironment and immunotherapeutic outcome in CLDN18.2-positive gastric cancer. *BMC Med*. 2022;20:223.

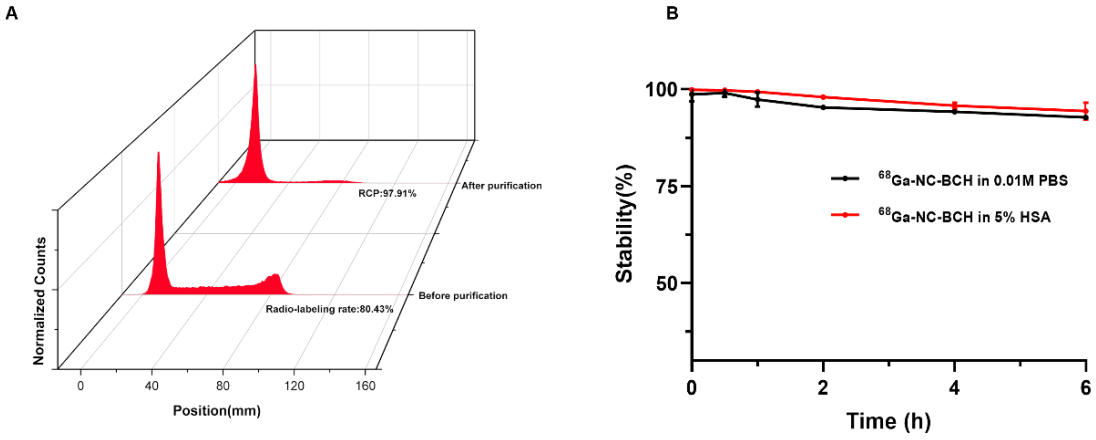
Figures.



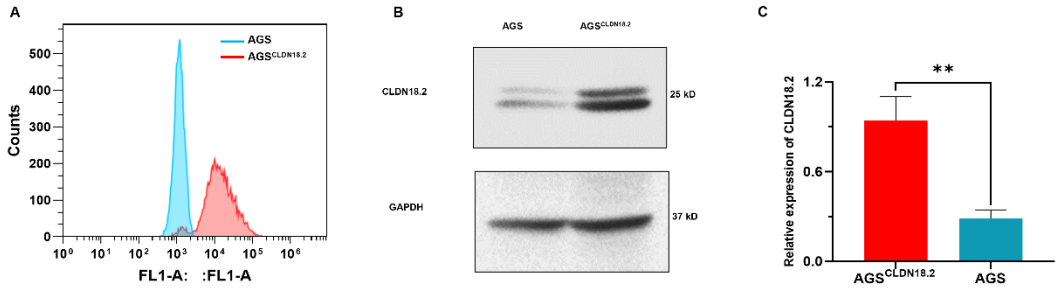
Supplemental Figure 1. (A) MALDI-TOF-MS of ACN376. (B) MALDI-TOF-MS of ACN376-G4C. (A) MALDI-TOF-MS of NOTA-ACN376. (D) Non-reducing SDS-PAGE characterization.



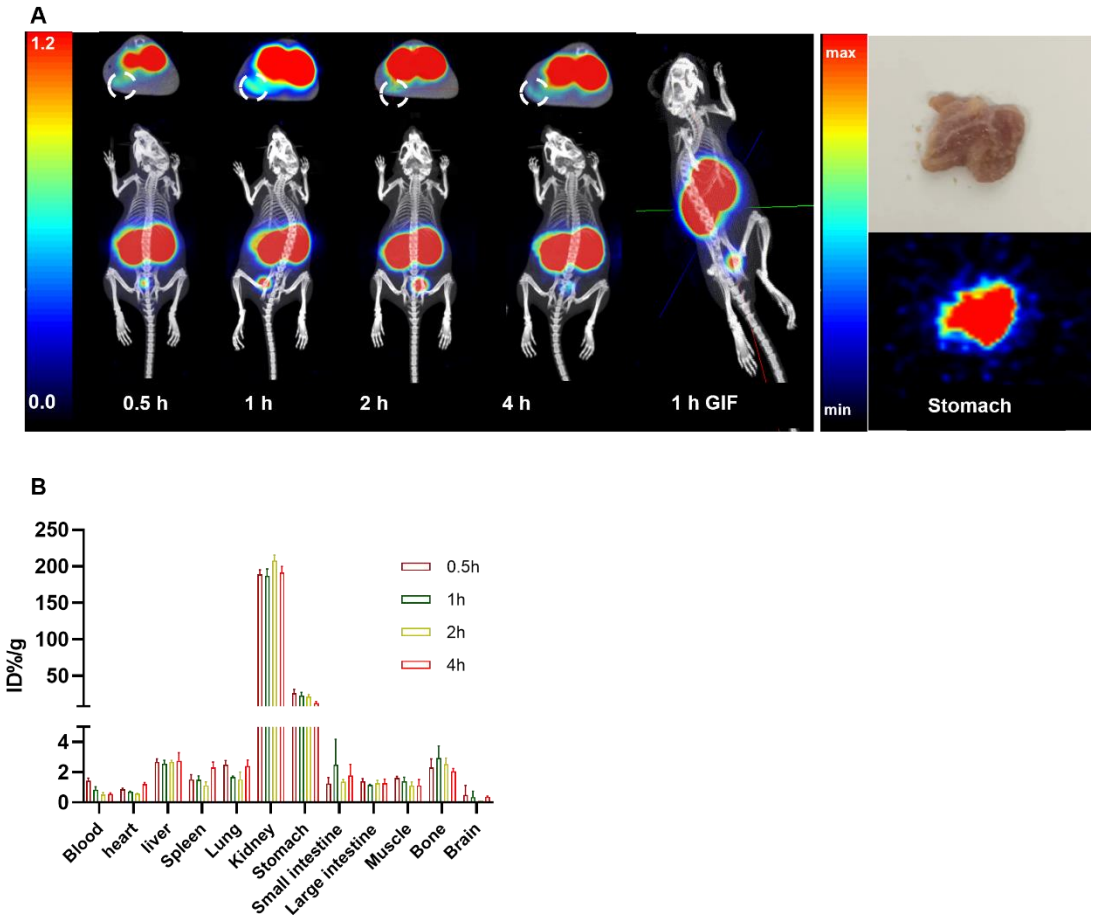
Supplemental Figure 2. (A) Synthesis of ^{68}Ga-NC-BCH. (B) Binding affinity assay of ^{68}Ga-NOTA-ACN376 to CLDN18.2 protein, the K_d value is 27.85 nmol/L. (C) Cellular uptake of ^{68}Ga-NOTA-ACN376 in AGS$^{CLDN18.2}$ and AGS cells.



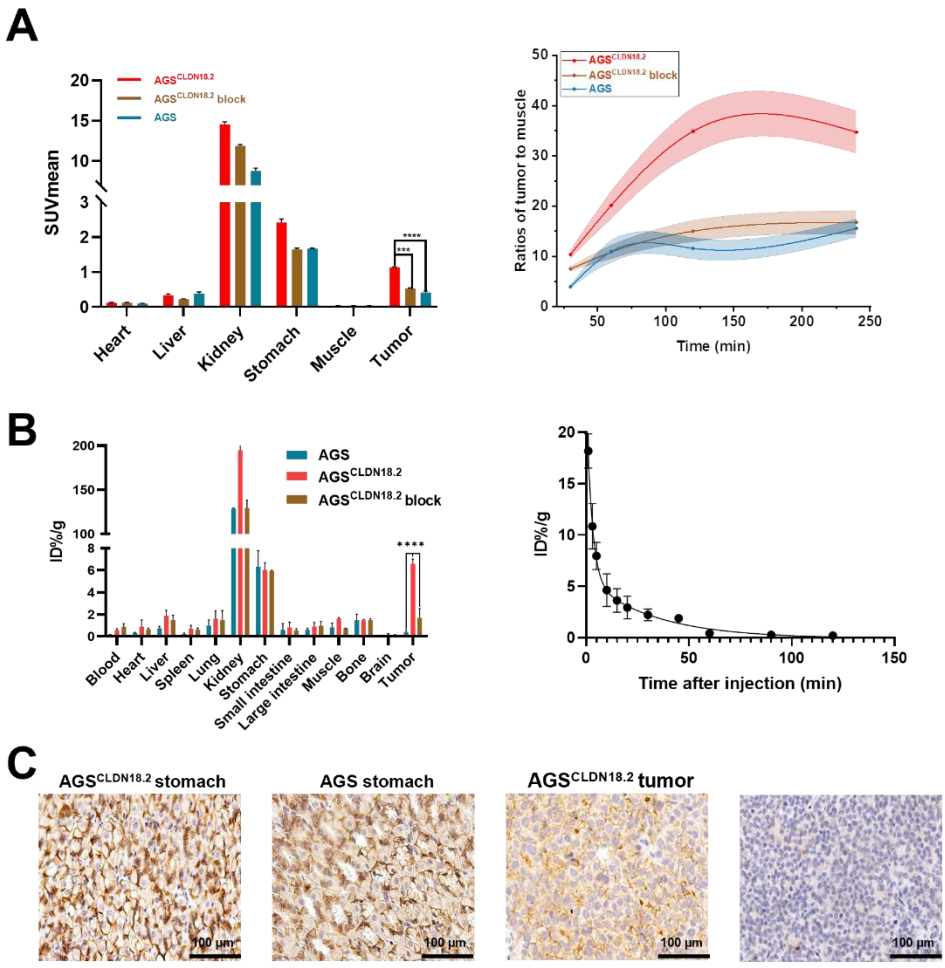
Supplemental Figure 3. Radio-TLC of ^{68}Ga-NC-BCH before and after purification and in vitro stability of ^{68}Ga-NC-BCH.



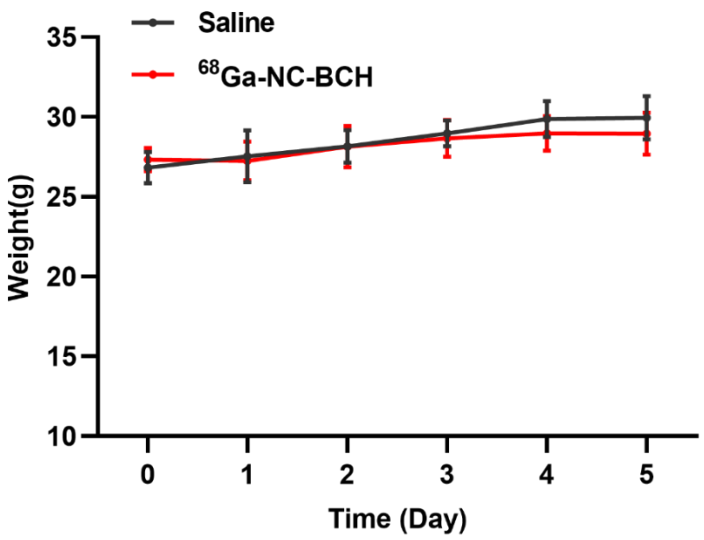
Supplemental Figure 4. (A) Flow cytometry histogram of AGS$^{CLDN18.2}$ and AGS cells. (B) Western blot result of mesothelin expression in the AGS$^{CLDN18.2}$ and AGS cell lines. (C) Relative expression of CLDN18.2 in the AGS$^{CLDN18.2}$ and AGS cells (results are shown as mean ± SD, n = 3).



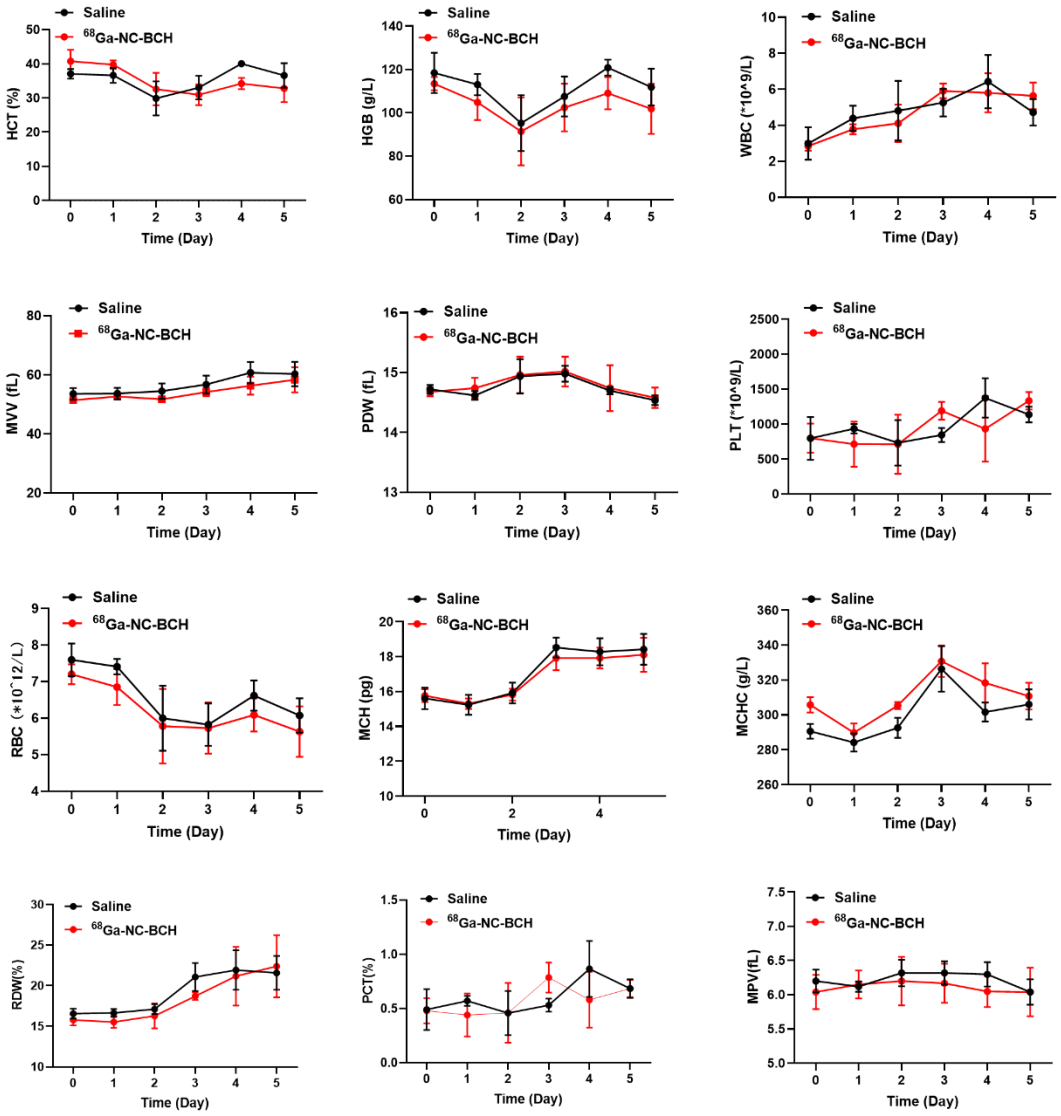
Supplemental Figure 5. ^{68}Ga-NC-BCH PET images of KM mice and biodistribution of KM mice post injection 0.5, 1, 2 and 4 h.



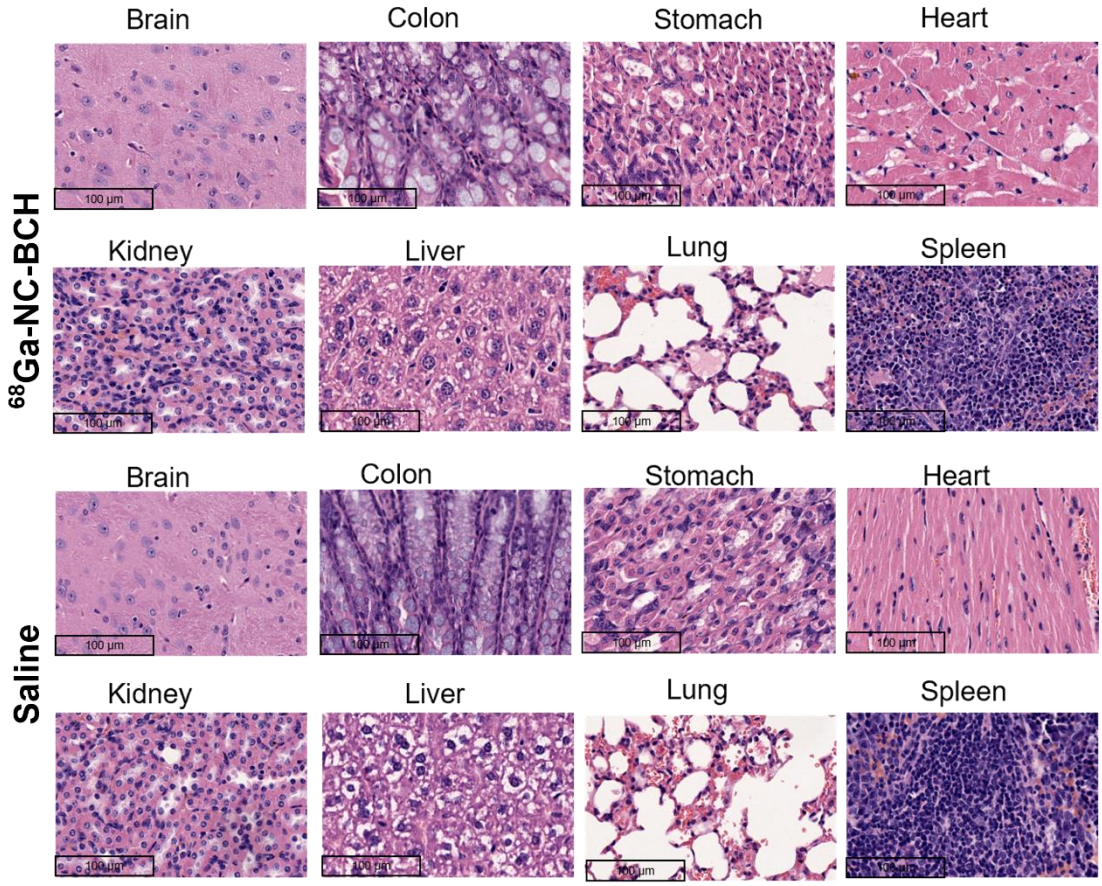
Supplemental Figure 6. (A) SUVmean of different experimental group mice in organs at 1h and the ratios of tumor/muscle (T/M) at each point post injection. (B) Biodistribution of ^{68}Ga-NC-BCH in AGS$^{CLDN18.2}$ and AGS tumor models post injection 2 h and pharmacokinetics study of ^{68}Ga-NC-BCH in KM mice. (C) IHC analysis of CLDN18.2 expression in AGS$^{CLDN18.2}$ Stomach (+++), AGS Stomach (+++), AGS$^{CLDN18.2}$ tumor (++) and AGS tumor (-).



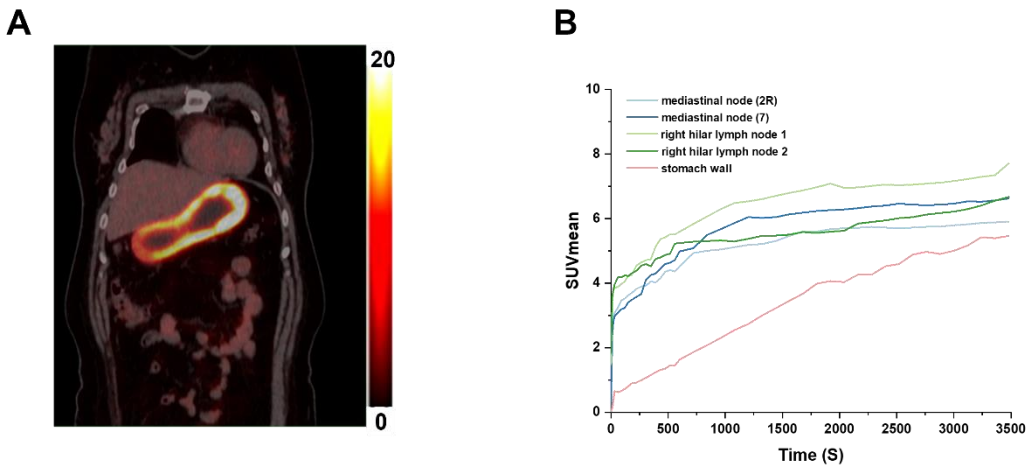
Supplemental Figure 7. Body weight of test group and control group, n = 6 for ^{68}Ga-NC-BCH treatment group and n = 4 for saline treatment group.



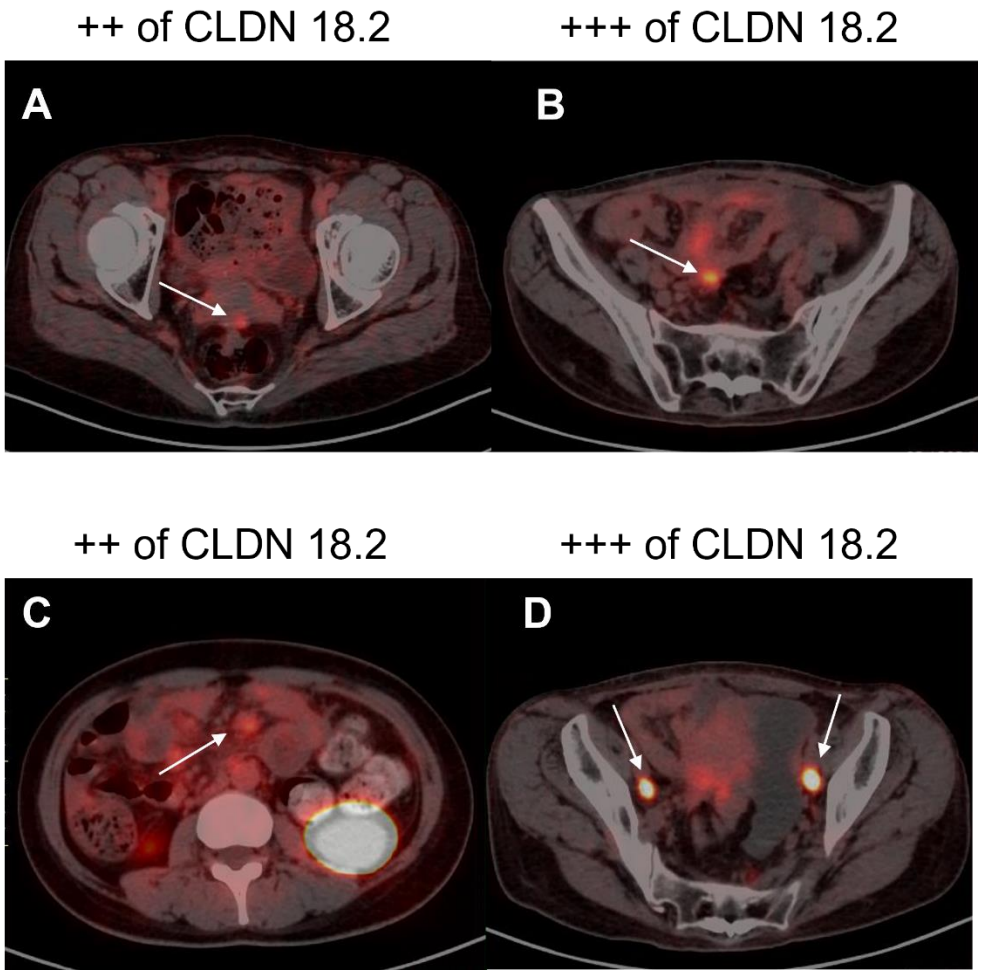
Supplemental Figure 8. Blood routine safety test. Experimental group vs control group.



Supplemental Figure 9. H&E staining of main organs taken from test group and control group



Supplemental Figure.10 (A) The gastric mucosal exhibits a diffuse high physiological uptake of CLDN18.2. (B) The dynamic changes of the SUVmean of mediastinal metastatic lymph nodes and stomach wall.



Supplemental Figure. 11 (A-B) The imaging of metastatic peritoneum at CLDN 18.2(++) patient No.3, CLDN 18.2(+++) No. 10. (C-D) The imaging of metastatic lymph node at CLDN18.2(++) patient No.6, CLDN 18.2(+++) No. 10.

Table

Supplemental Table 1. Quality control of ^{68}Ga-NC-BCH

Parameter	QC Specification	QC Result
Appearance	Clear, Colorless	Pass
Volume	1.0-3.0 mL	2.5 mL
pH	5.0-8.0	7.2
Radiochemical purity	>95%	>99%
Endotoxins	<15 EU/mL	Pass
Sterility	Sterile	Pass
Specific Activity	13.5−100 GBq/μmol	24.7 GBq/μmol

Supplemental Table 2. Blood biochemical safety test of ^{68}Ga-NC-BCH

Parameter	Reference range	^{68}Ga-NOTA-ACN376	Saline
ALT	25-500(U/L)	34.5	44.3
AST	25-500(U/L)	190.7	298.4
TBIL	5.0-680.0(umol/L)	3.5	2
UREA	0.90-35.70(mmol/L）	5.8	2.6
CREA	30-1500（μmol/L）	66	87.6
UA	50-2380（μmol/L)	32	41

Supplemental Table 3. Organ-absorbed dose and effective dose estimated from whole-body 68Ga-NC-BCH PET imaging

	Absorbed dose (mGy/MBq)			Mean	SD
Target Organ	Patient 02	Patient 03	Patient 05		
Adrenals	3.01E-02	1.05E-02	5.68E-02	3.25E-02	0.018976
Brain	2.51E-03	2.58E-03	1.55E-03	2.21E-03	0.00047
Breasts	3.03E-03	2.74E-03	1.14E-02	5.72E-03	0.004016
Esophagus	1.13E-02	1.13E-02	1.37E-02	1.21E-02	0.001131
Eyes	1.81E-03	6.61E-04	1.07E-02	4.39E-03	0.004486
Gallbladder Wall	4.18E-02	3.73E-02	2.89E-02	3.60E-02	0.005346
Left colon	1.61E-02	1.52E-02	1.81E-02	1.65E-02	0.001212
Small Intestine	1.45E-02	1.24E-02	1.73E-02	1.47E-02	0.002007
Stomach Wall	1.28E-01	1.36E-01	1.74E-02	1.32E-01	0.004
Right colon	1.33E-02	1.14E-02	1.77E-02	1.41E-02	0.002639
Rectum	4.72E-03	3.02E-03	1.41E-02	7.28E-03	0.004872
Heart Wall	2.54E-02	8.55E-02	2.49E-02	4.53E-02	0.02845
Kidneys	2.15E+00	1.91E+00	8.65E-01	1.64E+00	0.557858
Liver	5.23E-02	4.41E-02	1.65E-02	3.76E-02	0.015314
Lungs	6.14E-02	3.01E-02	8.68E-03	3.34E-02	0.021648
Ovaries/Prostate	5.54E-03	4.13E-03	1.48E-02	8.16E-03	0.004733
Pancreas	3.48E-02	2.70E-02	1.42E-02	2.53E-02	0.008492
Salivary Glands	1.58E-03	7.87E-04	1.14E-02	4.59E-03	0.004827
Red Marrow	1.09E-02	7.82E-03	1.20E-02	1.02E-02	0.001769
Osteogenic Cells	5.05E-02	1.42E-02	1.23E-02	2.57E-02	0.017577
Spleen	1.45E-01	9.39E-02	4.45E-02	9.45E-02	0.041031
Thymus	7.57E-03	6.99E-03	1.29E-02	9.15E-03	0.00266
Thyroid	2.32E-02	3.46E-02	1.90E-02	2.56E-02	0.006591
Urinary Bladder Wall	3.92E-02	1.93E-02	1.73E-02	2.53E-02	0.009886
Uterus	5.30E-03	3.76E-03	1.46E-02	7.89E-03	0.004788
Total Body	2.31E-02	1.80E-02	1.81E-02	1.97E-02	0.002381
Effective Dose (mSv/MBq)	5.69E-02	4.83E-02	2.14E-02	4.22E-02	0.015121