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[^{161}Tb]Tb-Thz-Phe-D-Trp-Lys-Thr-DOTA: a potential radiopharmaceutical for the treatment of neuroendocrine tumors

Anzhelika O. Fedotova, Bayirta V. Egorova, Gleb Yu. Aleshin, Lyubov S. Zamurueva, Ramiz A. Aliev, Galina A. Posypanova, Anna B. Priselkova, Anton V. Kolotaev, Derenik S. Khachatryan, Vasily N. Osipov and Stepan N. Kalmykov

Materials

Thz-Phe-D-Trp-Lys-Thr-DOTA (Figure S1) conjugate was synthesized as described earlier [A. O. Fedotova, B. V. Egorova, G. A. Posypanova, N. A. Titchenko, D. S. Khachatryan, A. V. Kolotaev, V. N. Osipov and S. N. Kalmykov, *J. Pept. Sci.*, 2021, **27**, e3361]. A 0.002M of Thz-Phe-D-Trp-Lys-Thr aqueous stock solution was prepared. Deionized Milli-Q water (18.2 MΩ cm; Millipore) was used in all reactions. Fetal bovine serum (FBS) was purchased from HyClone. ^{152}Eu was purchased from Ritverc Gmbh and was rinsed with 0.07 M HCl to obtain an MCl_3 solution. The specific activity was 75 kBq/ml.

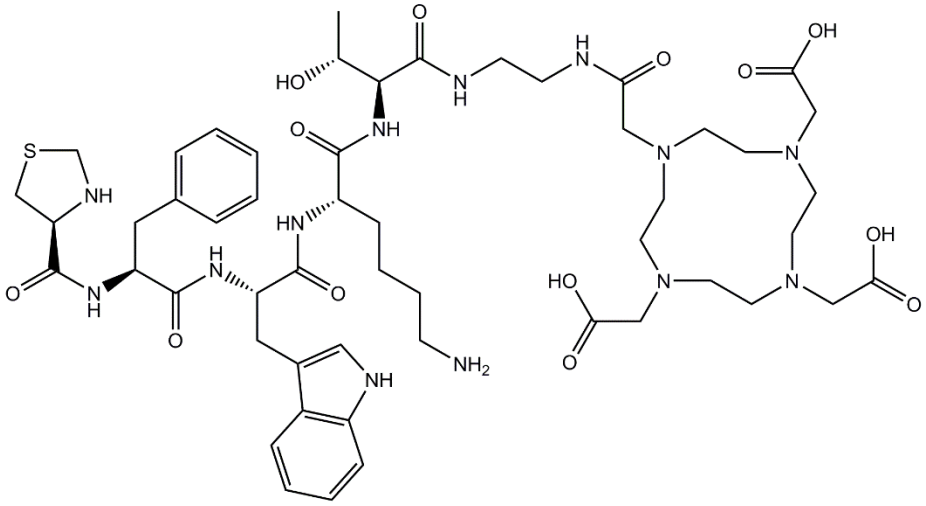


Figure S1. Thz-Phe-D-Trp-Lys-Thr-DOTA structure.

The quality control of the labeled complexes was performed using TLC on cellulose on Al (Fluka, Germany) and on silica gel on Al (Sigma-Aldrich, Germany). The autoradiography was carried out with a Perkin Elmer Cyclone Plus Storage Phosphor System and associated software. All

samples including TLC plates were measured by gamma spectrometry with an HPGe-detector GR3818 Canberra Ind.

IMR-32 human neuroblastoma cells were obtained from the Russian collection of cell cultures (St. Petersburg, Russia). The cells were maintained in culture flasks (Corning, USA) in a DMEM medium (Gibco, USA), supplemented with 10% FBS (HyClone, USA) and 50 μg/mL gentamicin (HyClone, USA) in a CO_2 incubator at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged twice a week using a trypsin-EDTA solution.

Methods

Production and isolation of ^{161}Tb

Gadolinium oxide targets of natural isotopic composition (chemical purity 97%, main impurity Y < 2%, Dy < 0.01%, Tb < 0.07% according to ICP-MS results) were prepared by sedimentation. 2.4 ml of acetone and 13 μl of collodium (1–2% by weight of dinitrocellulose) were added to 40.7 mg of Gd_2O_3 powder. The suspension was evaporated on a 29.64 μm thick aluminum support. The thickness of target was determined by weighing as 14.2675 mg/cm^2. The Gd_2O_3 layer visually looked homogeneous, had no defects, and was firmly adhered to the substrate. The surface of target was additionally covered with a thin 8.3 μm aluminum foil in order to prevent them from sticking together during irradiation. A target assembly, including Gd_2O_3 target, as well as Al (8.3 μm), and Ti (2.16 μm) monitors, was irradiated on a deutron beam from the U-120 cyclotron of the Skobeltsyn Institute of Nuclear Physics (Lomonosov Moscow State University) for 120 min by a deuteron beam with an energy of 13,3 MeV and an average current of 1 μA.

To control the energy in the stack of targets, there were aluminum and titanium monitors, the activity of which was determined.

HP Ge detector Canberra GR3819 (USA) with a corrugated aluminum entrance window was used for radioactivity measurements. Efficiency calibration was performed using a certified ^{152}Eu, ^{137}Cs and ^{60}Co gamma ray sources with a distance between the source and detector of 1 cm. Detector relative efficiency was 12.8% and 1.7 keV resolution for 1332 keV line. The detector was located inside a 10 cm thick lead passive shield. The spectrum was processed using the SpectraLine software (LSRM, Russia).

The irradiated material (~20 mg Gd) was removed from the support and dissolved in 2–3 ml of concentrated HNO_3 with gentle heating. Then the solution was evaporated near dryness, and the concentration of HNO_3 was adjusted to 0.5 M. Aliquots from stock solution were used for further experiments.

1 g of LN resin (di(2-ethylhexyl)phosphoric acid (HDEHP) on an inert support) with a grain size of 100–150 μm (TrisKem International) was preequilibrated with 0.5 M HNO_3 and transferred into a 7 cm high and 7 mm i.d. column. The column was washed with 0.5 M HNO_3 and aliquot 570 μl of stock solution was loaded onto the column. Then, 0.5 M HNO_3, 0.8 M HNO_3, and, finally, 2.7 M HNO_3 were passed through the column sequentially, washing out Gd and Tb. 1 ml fractions were collected and the content of radionuclides was monitored by gamma-ray spectrometry. Gadolinium was identified by the 363.5 keV (11.78%) ^{159}Gd peak, terbium by the 105 keV (25,1%) of ^{155}Tb and 199.2 keV (41%) of ^{156}Tb peaks. The combined fraction of ^{161}Tb from was evaporated to 1 mL and the long exposition gamma spectrum was measured. The specific activity was 9 kBq/mL.

Labeling of Thz-Phe-D-Trp-Lys-Thr-DOTA

Labeling Thz-Phe-D-Trp-Lys-Thr-DOTA with ^{152}Eu or ^{161}Tb was performed by mixing different volumes of the Thz-Phe-D-Trp-Lys-Thr-DOTA stock-solution with 40 μl of a stock of radionuclide solution in 0.1 M of HCl and 20 μl of 10 μM $Eu(ClO_4)_3$ (for ^{152}Eu) and 20 μL of 10μM $Tb(NO_3)_3$ (for ^{161}Tb). The metal:ligand ratio was 1:500. The mixtures were kept in a heating chamber at 90°C. The labeling reaction was optimized by varying the incubation time, concentration of Thz-Phe-D-Trp-Lys-Thr-DOTA, and pH of the reaction mixtures. For *in vivo* experiments, solutions were heated for 1 h at 90°C. The pH factor for [^{152}Eu]Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA and [^{161}Tb]Tb-Thz-Phe-D-Trp-Lys-Thr-DOTA complexes was adjusted by the addition of 0.2 M of Na_2CO_3 to the mixture of 0.1 mM of Thz-Phe-D-Trp-Lys-Thr-DOTA. In kinetic experiments, the labeling reactions were quenched by the addition of 10 μl of 8 mM DTPA to 50 μl of the sample.

Quality control

Thin layer chromatography (TLC) was used for quality control of labeled complexes. Cellulose on Al TLC plates were used and developed by 0.01 M EDTA, pH 6.0 and silica gel on Al TLC plates were used and developed by 0.1 M Na_3Cit, pH 5.0 for both [^{152}Eu]Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA and [^{161}Tb]Tb-Thz-Phe-D-Trp-Lys-Thr-DOTA complexes. The R_f values for free M^{3+}, M-DTPA, M-Thz-Phe-D-Trp-Lys-Thr-DOTA were 1; 1 and 0 respectively in all the systems selected. The plates were cut in 2 parts. These parts were measured by gamma-spectrometry enabling to determine the labeling yield. The activity was quantified by the 25.7 keV gamma emission of ^{161}Tb, and 121 keV of ^{152}Eu.

Stability studies

All M-Thz-Phe-D-Trp-Lys-Thr-DOTA complexes of radiochemical purity 95-98% were used for stability studies. Stability studies in serum were performed by the addition of 20 μl of free M^{3+} (blank), ^{152}Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA, or ^{161}Tb-Thz-Phe-D-Trp-Lys-Thr-DOTA to 2 ml of fetal bovine serum, and the mixture was incubated at 37°C for 24 h. 100 μl aliquots were taken at appropriate time intervals (0, 30, 60, 240 min and 24 h) and treated with 300 μl of ethanol. Samples were cooled to 2-4°C and centrifuged for 5 minutes at 4000g to precipitate serum protein. 250 μl of supernatant was separated and measured by gamma-spectrometry. The precipitate was washed with 1 ml of ethanol and measured. The activity of the supernatant was compared with the initially added activity and serum protein bound fraction was determined.

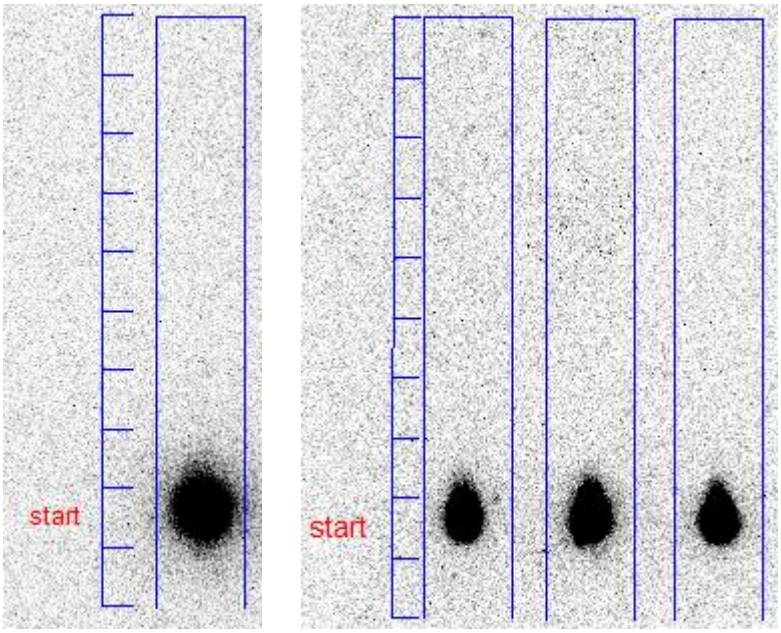
In vivo experiments

All in vivo experiments were performed in accordance with the EU Directive 2010/63/EU for animal experiments and were approved by the Bioethics Commission of M. V. Lomonosov Moscow State University, meeting no. 128-a, 31.05.2021, protocol no. 131-d. For the in vivo experiments, male Nu/j mice were used (weight, 32 ± 4 g). The mice were housed in a 12 h light/dark cycle with access to water and food ad libitum. Mice were injected subcutaneously with 200 μl of cell suspension in PBS ($1 \cdot 10^7$ cell per mouse). Tumor growth was observed for 5 weeks. Solutions of [^{152}Eu]$EuCl_3$, [^{152}Eu]Eu-DOTA-TATE and [^{152}Eu]Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA with a ligand concentration of $1 \cdot 10^{-5}$ M were buffered at pH 5.5 with 0.5 M MES at room temperature and diluted in a sterile isotonic solution. The radiochemical yield according to TLC reached 95%. The mice were administered with 3 kBq in 100 μL of solution via intravenous injection. For "blocking" experiments, a 10-fold excess of octreotide was preliminarily injected. At 6 h after injection, the mice (4 per data point) were euthanized by cervical dislocation and decapitation. Blood was collected immediately after euthanasia and mixed with heparin solution (100 μL). Urine was collected from the bladder with a syringe after autopsy. The major organs were harvested, washed free of blood and peritoneal liquid using 0.9% NaCl solution, and wet-weighed, and the radioactivity of each organ was measured by gamma spectrometry. The percentage injected dose per gram (% ID/g) was determined for each tissue. The values presented are means with standard deviation for each tissue.

Results

Table S1. The percentage injected dose per gram (ID/g, %), n=4, P=95%.

	[^{152}Eu]Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA	[^{152}Eu]Eu-DOTA-TATE	[^{152}Eu]$EuCl_3$
tumor	0.234±0.313	0.367±0.481	0.082±0.183
heart	0.060±0.017	0.046±0.005	0.135±0.165
lungs	0.122±0.091	0.093±0.051	1.211±1.329
liver	0.271±0.172	0.210±0.090	2.476±3.415
kidney	9.724±6.22	6.467±3.072	0.101±0.201
bone	0.399±0.193	0.109±0.018	0.282±0.205
spleen	0.119±0.071	0.129±0.046	1.827±3.091
pancreas	0.053±0.015	0.138±0.035	0.180±0.191
brain	0.019±0.017	0.026±0.009	0.075±0.101
blood	0.246±0.447	0.049±0.035	0.515±0.878
tumor to blood	1.757±3.389	6.759±4.260	0.310±0.301
tumor to liver	0.743±0.663	1.644±1.571	0.294±0.823
tumor to kidney	0.021±0.021	0.053±0.048	1.897±1.957



a) b)

Figure S2. Digital radiography of TLC plates: a) [^{152}Eu]Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA before the injection, b) urine samples from 3 mice.

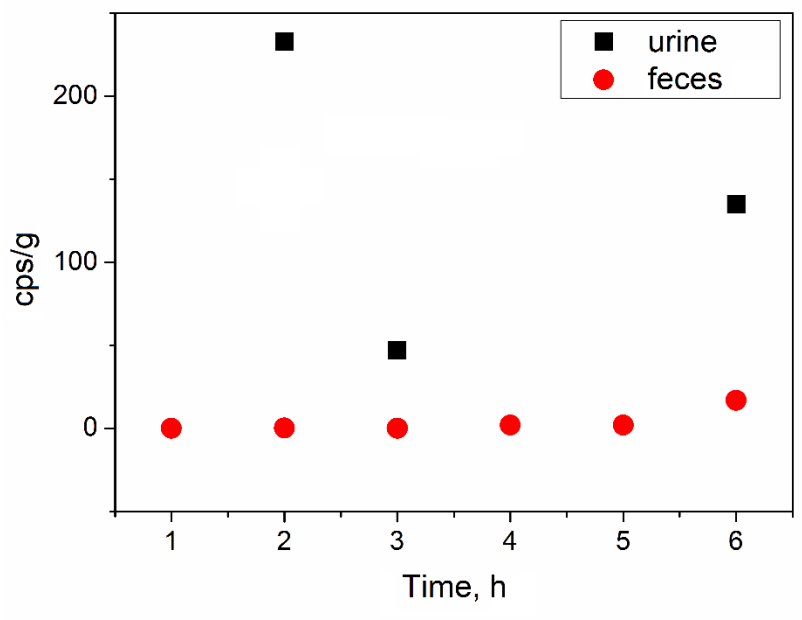


Figure S3. Counting rate of the collected excretion products from mice administered with [^{152}Eu]Eu-Thz-Phe-D-Trp-Lys-Thr-DOTA.