Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Synthesis, Proteolytic Stability, and In vitro Evaluation of DOTA Conjugated p160 Peptide Based Radioconjugates: [^{177}Lu]Lu-DOTA-p160

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Table of Contents

Figures:

Reagents

All chemicals and solvents were either of analytical or HPLC grade and used without further purification. Fmoc amino acid derivatives were purchased from Iris Biotech (Marktredwitz, Germany) and Novabiochem (Läufelfingen, Switzerland). 1-Hydroxybenzotriazole (HOBt), (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) (TBTU) and 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxymethyl-linked polystyrene (Rink amide) were obtained from Novabiochem (Läufelfingen, Switzerland). DOTA tris-*tert*-butyl ester (DOTA-(^{t}Bu)$_3$), piperdine and trifluoroacetic acid (TFA) were procured from Sigma. N,N-Diisopropylethylamine (DIPEA) was obtained from Alfa Aesar and thioanisole form Fluka. Dichloromethane (DCM) and N,N-dimethylformamide (DMF) were Biosolve (Valkenswaard, Netherlands) products. Diisopropylcarbodiimide (DIC) was obtained from Iris Biotech (Marktredwitz, Germany).

Electrospray Ionization (ESI): Mass spectra were measured on Bruker Esquire 6000 (ESI) instrument. The solvent flow rate for the ESI measurements was 4 mL min^{-1} with a nebulizer pressure of 10 psi and a dry gas flow rate of 5 L min^{-1} at a dry gas temperature of 300 °C. Various analytical and preparative HPLC studies were performed on a Merck-Hitachi system with Phenomenex Jupiter® 4 µm Proteo 90 Å, LC Column 150 x 2 mm.

Circular dichroism (CD): The circular dichroism (CD) spectra were recorded on a JASCO model J715 spectropolarimeter over a wavelength range of 320-190 nm in a nitrogen atmosphere. Samples at a concentration of approximately 50 µg/mL were measured in a solution of distilled water containing 10% trifluoroethanol (TFE).

For enzymatic studies, sterile water was used for preparing buffers. Trypsin from porcine pancreas (lyophilized powder, EC 3.4.21.4), pronase E from Streptomyces griseus (lyophilized powder, EC 3.4.24.31), elastase from hog pancreas (lyophilized powder, EC 3.4.21.36) and pepsin from hog stomach (lyophilized powder, EC 3.4.23.1) were purchased from Sigma- Aldrich. Fresh human plasma and serum was obtained from Lonza Bioscience.

Cell Cultures: The following media and supplements were used for cell cultures: HeLa cells were maintained in RPMI-1640 (with L-glutamine and $NaHCO_3$) culture medium (Sigma) containing 10% fetal calf serum, 50 µg/mL penicillin, and 0.05 g/mL streptomycin in a humidified CO_2 incubator (5% CO_2, 95% air). Monolayer cultures of normal embryonic kidney cells, HEK cells were maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 5 units/mL Penicillin and 0.05 g/mL streptomycin in a humidified CO_2 incubator (5% CO_2, 95% air). Growing cells were detached from the culture flasks using a trypsin 0.25% EDTA solution and the cell suspension was seeded on CELLSTAR® 96-well plates (Greiner bio-one, Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay were performed on Elisa reader from Biotek (Germany).

Experimental procedures

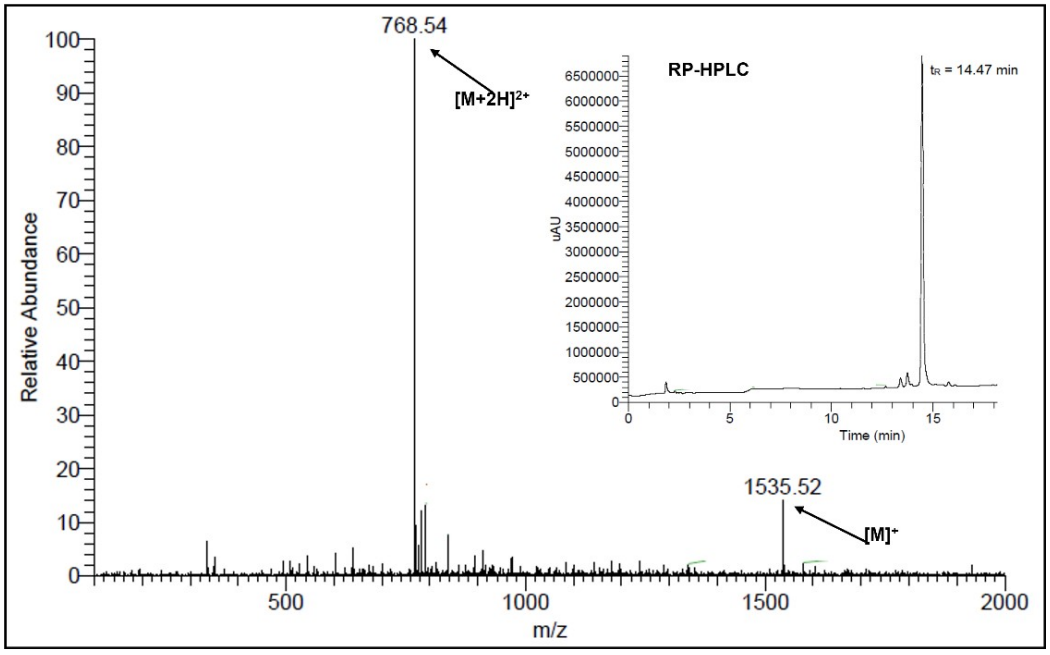
Synthesis of DOTA-p160 peptide derivatives

The peptides p160 (VPWMEPAYQRFL) and 3 derivatives, namely, p160-1 (EPAYQRFL), p160-2 (WMEPAYQR) and p160-3 (VPWMEPAY) were synthesized by automated solid-phase peptide synthesis (SPPS) on a rink amide resin (30 mg, resin loading 0.45 mmol g^{-1}) using the Fmoc/^{t}Bu-strategy on a multiple synthesizer (Syro, MultiSynTech, Bochum, Germany) as described elsewhere.[1] The peptides were cleaved from the resin by treatment with trifluoroacetic acid (90%) and a scavenger mixture containing thioanisole (7%) and 1,2-ethanedithiol (3%) for 3 h and precipitated by addition of ice-cold diethyl ether. Precipitation and multiple washing with diethyl ether yielded the crude peptides which were analyzed by (reversed-phase) RP-HPLC and ESI-MS. ESI ion trap measurements were performed on a Bruker HCT mass spectrometer. Analytical RP-HPLC was done on a Merck-Hitachi system with a Grace Vydac 218TP54 column (4.6 × 250 mm; 5 µm; 300 Å) using a linear gradient of 10-60% of acetonitrile/0.08% TFA in water/0.1% TFA over 15 min and a flow rate of 0.6 mL min^{-1}. Experimentally determined and calculated masses of the peptides were consistent as shown in Figure S1.

Table S1: Names, sequences, and molecular weights of synthesized p160 peptide derivatives.

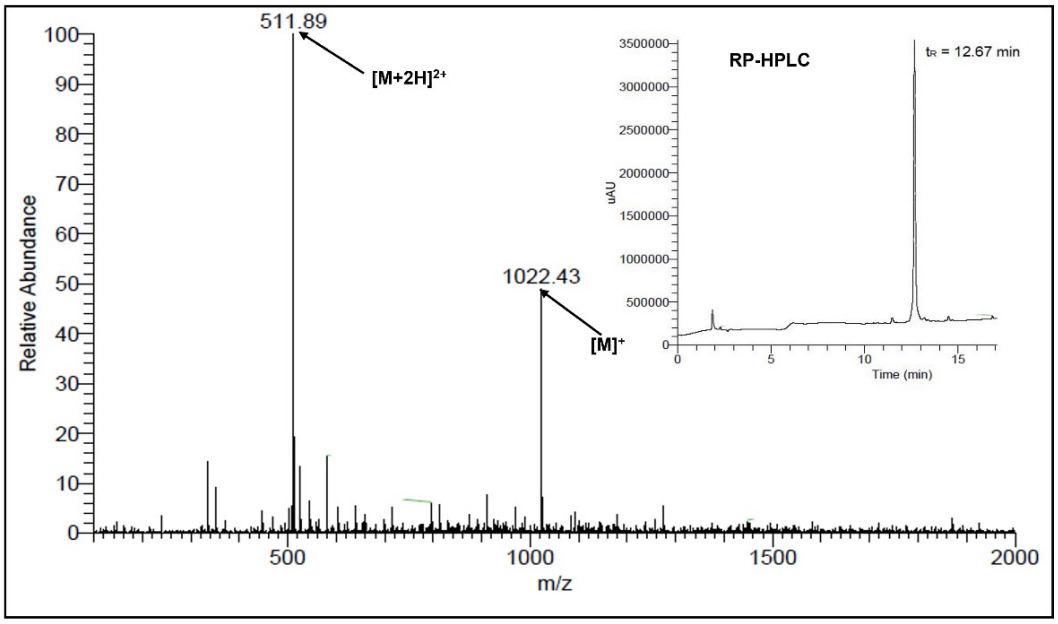
Peptide	Sequences	$MW_{calc.}$	$MW_{exp.}$	t_R [min]
p-160	VPWMEPAYQRFL	1535.83	1535.52	14.47
p-160-8-1	EPAYQRFL	1022.17	1022.43	12.67
p-160-8-2	WMEPAYQR	1079.24	1079.32	12.62
p-160-8-3	VPWMEPAY	991.18	991.28	13.27

A) ESI-MS: p160 peptide
($MW_{calcd.}$=1535.83)



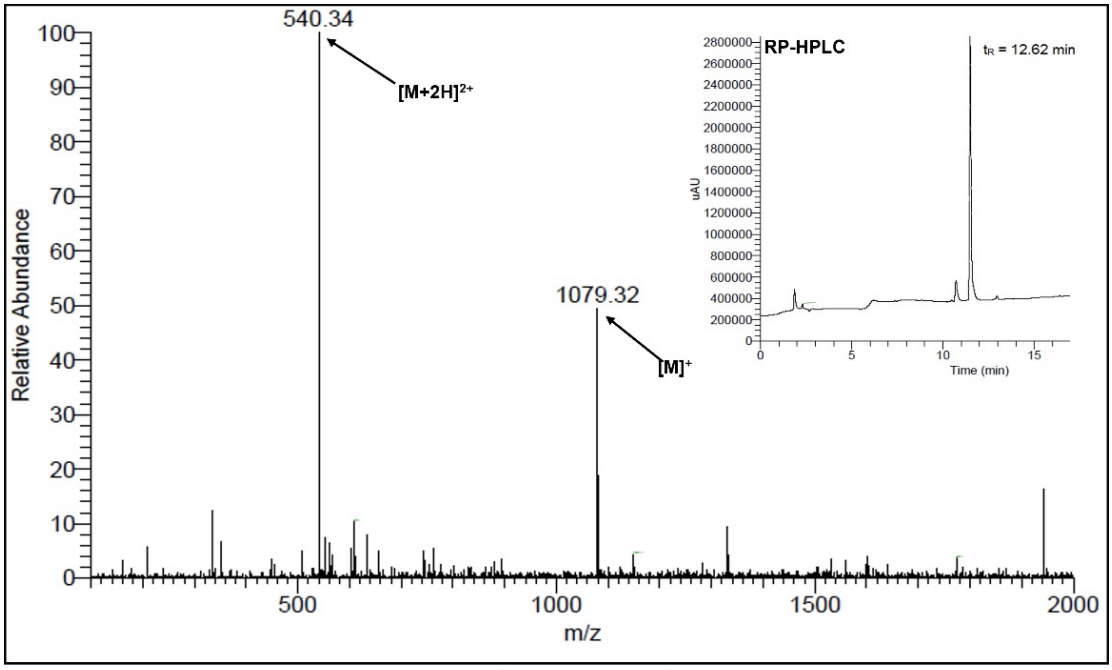
p160 peptide: Val-Pro-Trp-Met-Glu-Pro-Ala-Tyr-Gln-Arg-Phe-Leu

B) ESI-MS: p160-1 peptide
($MW_{calcd.}$=1022.17)



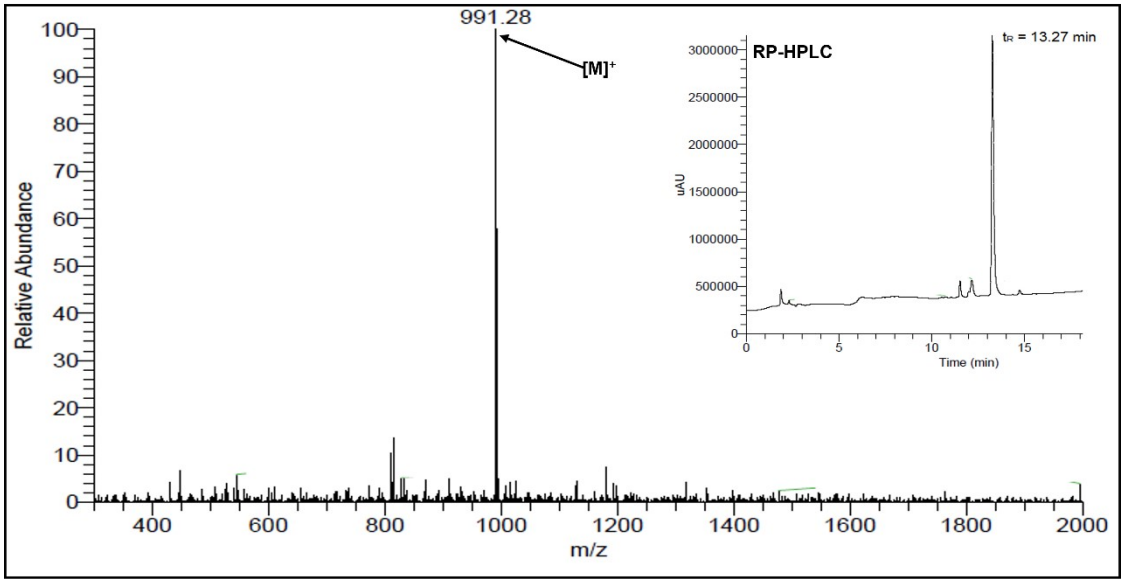
p160-8-1: Glu-Pro-Ala-Tyr-Gln-Arg-Phe-Leu

C) **ESI-MS: p160-2 peptide**
($MW_{calcd.}$=1079.24)



p160-8-2 Trp-Met-Glu-Pro-Ala-Tyr-Gln-Arg

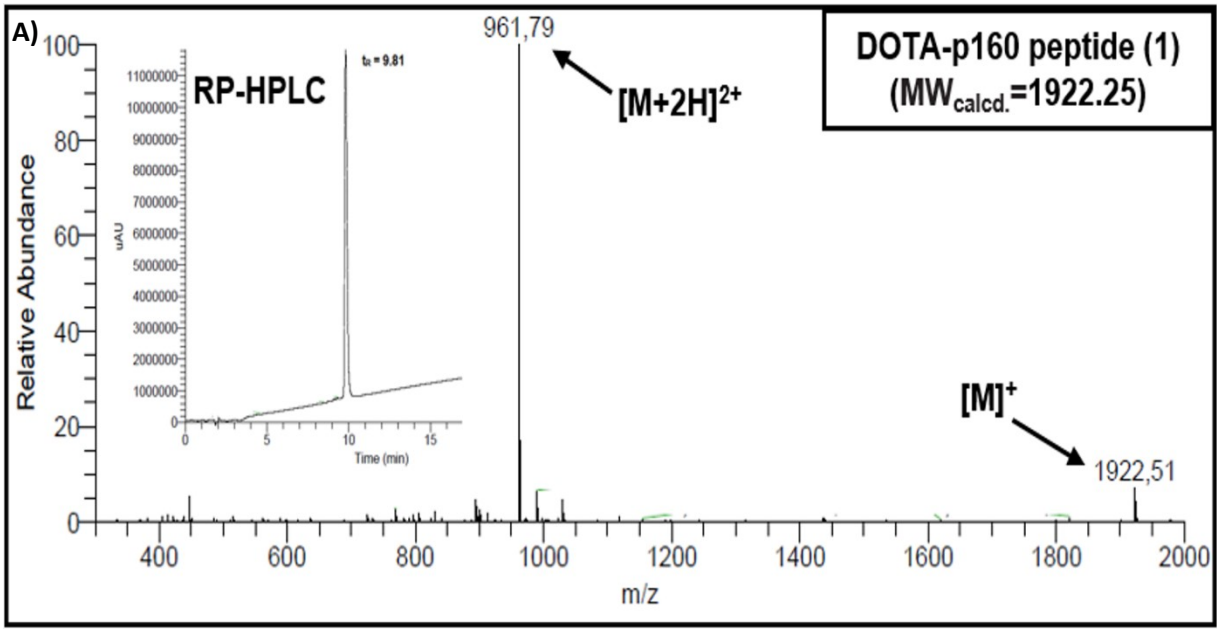
D) **ESI-MS: p160-3 peptide**
($MW_{calcd.}$=991.18)

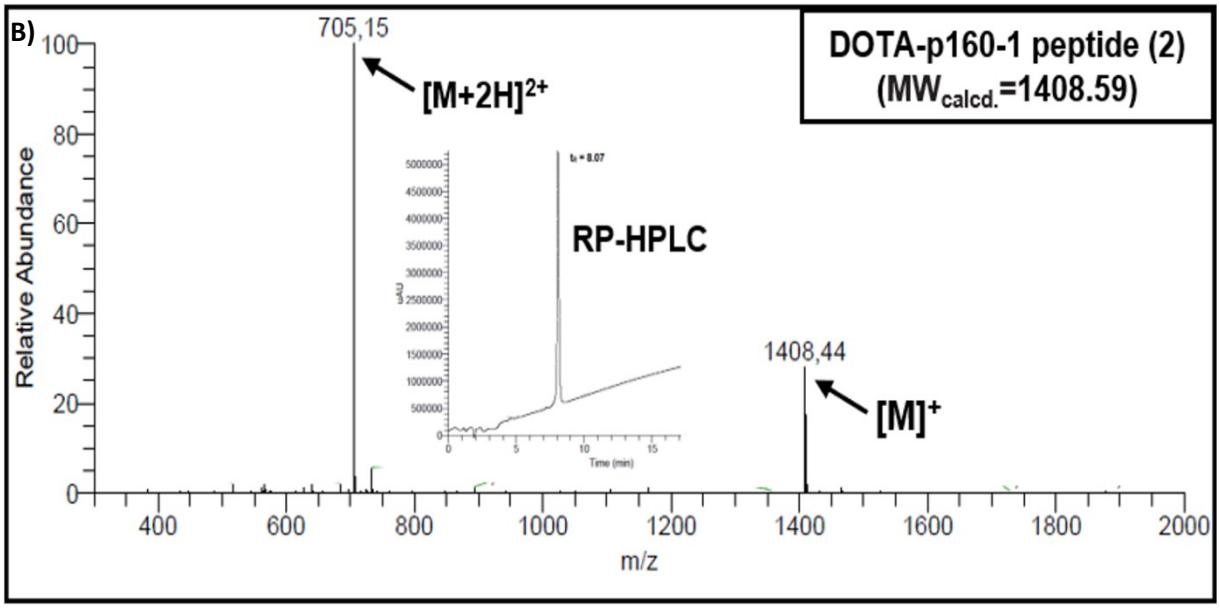


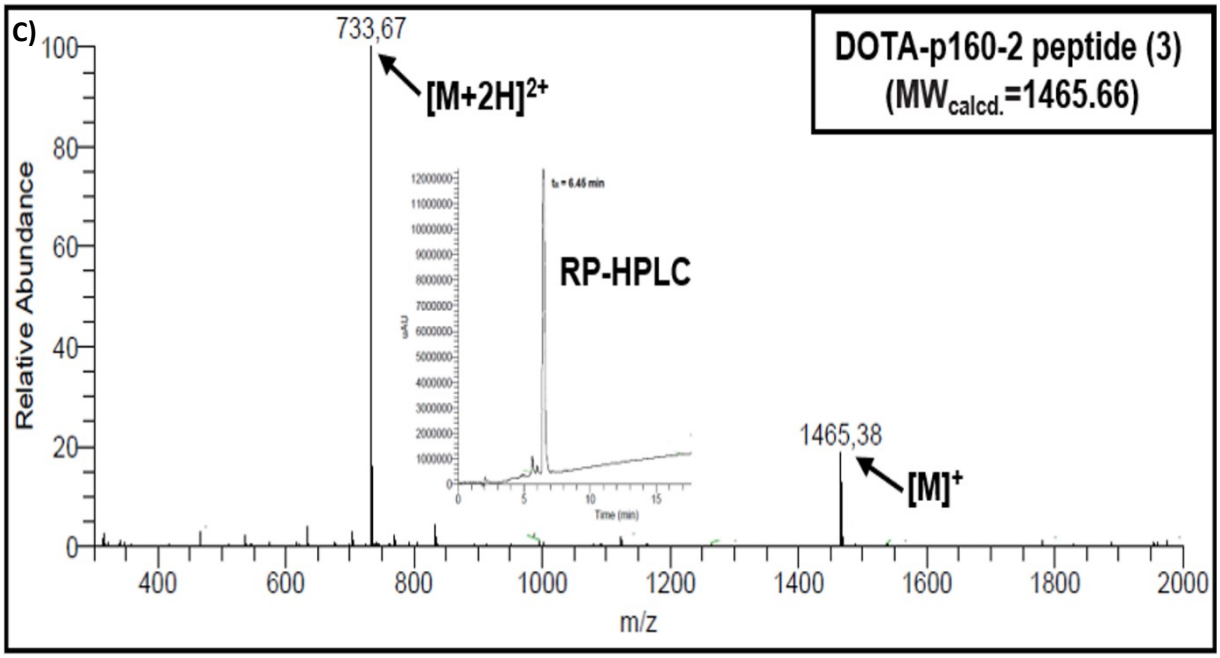
p160-8-3 Val-Pro-Trp-Met-Glu-Pro-Ala-Tyr

Figure S1. ESI-MS spectrum and RP-HPLC chromatograms of A) p160 peptide; B) p160-1; C) p160-2; D) p160-3

The p160 peptide and its derivatives were N-terminally coupled with DOTA using DOTA tris-*t*-butyl ester (Sigma Aldrich). The resin was swollen in anhydrous DMF (0.5 mL). DOTA-tris-*t*-butyl ester, [DOTA (*t*-Bu)$_3$] (3 equiv.) and Oxyma Pure (3 equiv.) were dissolved in DMF (50 µL). This solution was added to the pre-swollen resin and pre-incubated for 2 min. Subsequently, DIC (3.52 µL, 3 equiv) was added to the reaction mixture and it was shaken overnight at room temperature. The resin was washed with methanol (3 x 2 mL) and dichloromethane (3 x 2 mL) followed by drying for 15 minutes under vacuum. DOTA coupled p160 peptide derivatives were cleaved from the resin by treatment with TFA (90%) and a scavenger mixture containing thioanisole (7%) and 1,2-ethanedithiol (3%) for 4 h. The solution was filled in a falcon tube and ice-cold ether (10 mL) was added. The resin was washed with TFA (200 µL) and was added to above solution. The mixture was incubated for 30 min on ice and the precipitated DOTA-p160 peptide conjugates were collected as pellet by centrifuge spinning at 1150 rpm for 5 minutes. The supernatant liquid was decanted, the DOTA-p160 peptides were dissolved in water, precipitated with diethyl ether, and the centrifuge/wash procedure was repeated three times. The final precipitates were dissolved in water and lyophilized giving white amorphous fluffy solids. Crude DOTA coupled p160 peptide derivatives were purified by preparative RP-HPLC on a Shimadzu Chromatopac system using a stepwise gradient of 0–20% solvent B in A (Solvent A: 0.1% TFA/H_2O; Solvent B: 0.08% TFA/CH_3CN) in 10 min followed by 20%–40% solvent B in A in 50 min. Fractions corresponding to the desired products were collected and analyzed by analytical HPLC and ESI-MS (Figure S2). Pure fractions were combined and frozen at -80ºC followed by subsequent lyophilization.




B)
705,15
[M+2H]2+
DOTA-p160-1 peptide (2)
(MWcalcd.=1408.59)
RP-HPLC
1408,44
[M]+
Relative Abundance
m/z


C)
733,67
[M+2H]2+
DOTA-p160-2 peptide (3)
(MWcalcd.=1465.66)
RP-HPLC
1465,38
[M]+
Relative Abundance
m/z

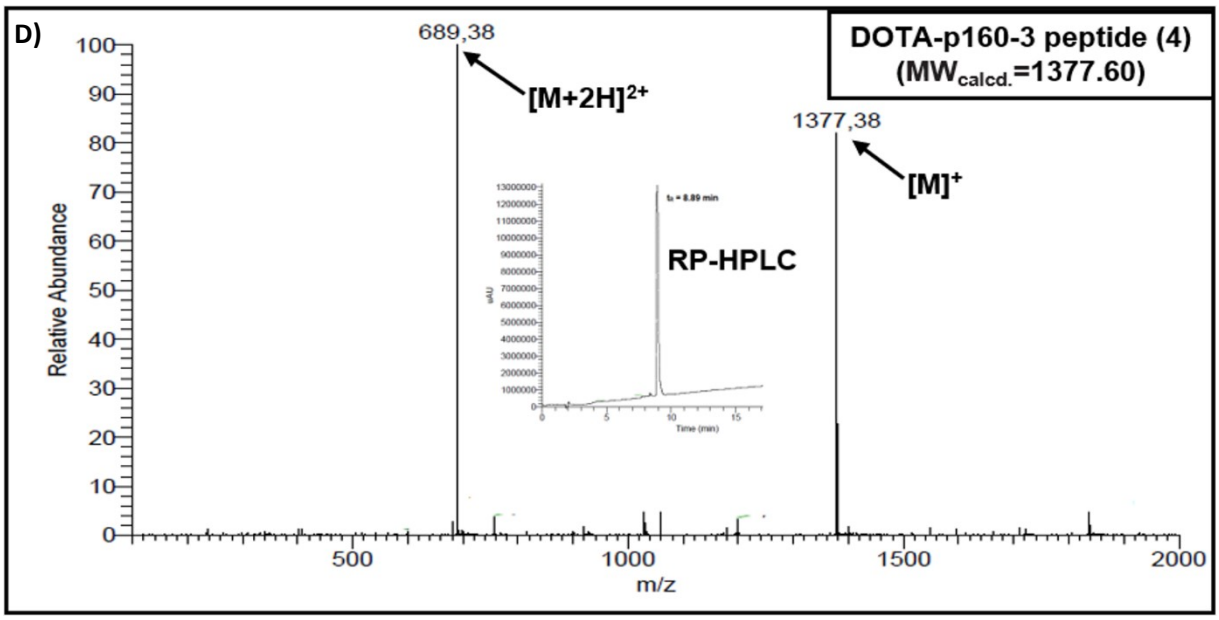
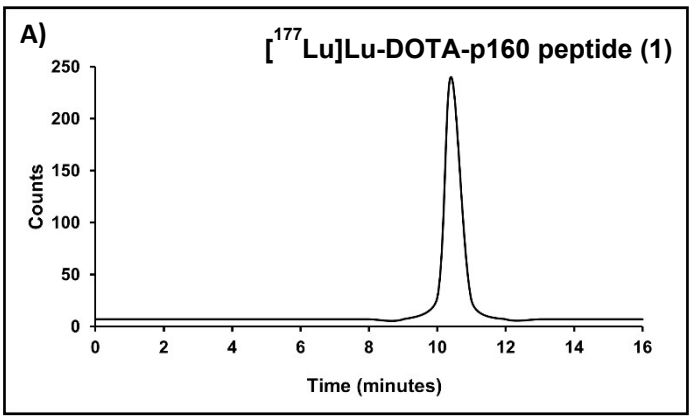


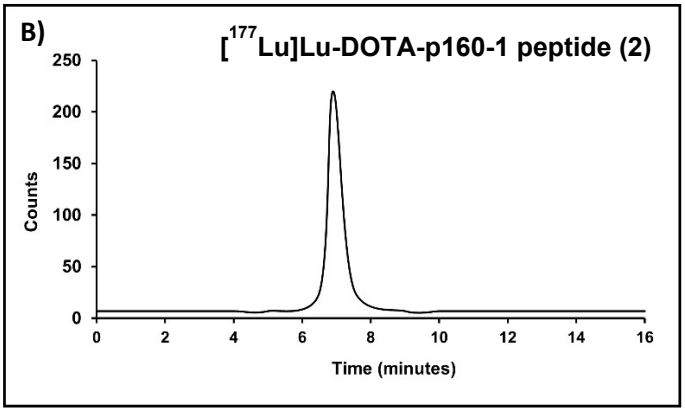
Figure S2. ESI-MS spectrum and RP-HPLC chromatograms of A) DOTA-p160 peptide **(1)**; B) DOTA-p160-1 **(2)**; C) DOTA-p160-2 **(3)**; D) DOTA-p160-3 **(4)**

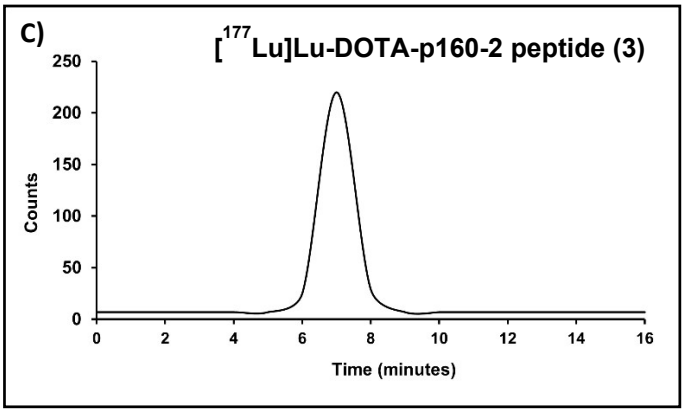
Radiolabeling

177-Lu was purchased from IDB Radiopharmacy (Netherlands).

To a solution containing DOTA-p160 peptides (1.25 µM) in sodium acetate buffer (pH 4.5) was added 10 µL of $^{177}LuCl_3$ (8.6 MBq). The reaction mixture was incubated at 80°C for 10 min, with continuous stirring. The reaction mixture was cooled to room temperature and the radiolabeling yield was determined by instant thin layer chromatography (ITLC). The radiochemical purity of the radiolabeled conjugates was assessed by RP-HPLC on a WellChrom HPLC-pump K-1001 equipped with a solvent organizer K-1500, a 4-Channel degasser K-5004 and a manual 6-port/3 channel injection valve with a sample loop of 20 µL. The compounds were analyzed using a Hypersil ODS C18 column (5mm, 250×4 mm) and gradient of 5%–95% solvent B (Solvent A: 1% TFA/H_2O; Solvent B: 1% TFA/CH_3CN) at a flow rate of 6 mL/min (Figure S3). All results were expressed as mean %SD of three experiments.


A)
[177Lu]Lu-DOTA-p160 peptide (1)
Counts
Time (minutes)


B)
[177Lu]Lu-DOTA-p160-1 peptide (2)
Counts
Time (minutes)


C)
[177Lu]Lu-DOTA-p160-2 peptide (3)
Counts
Time (minutes)

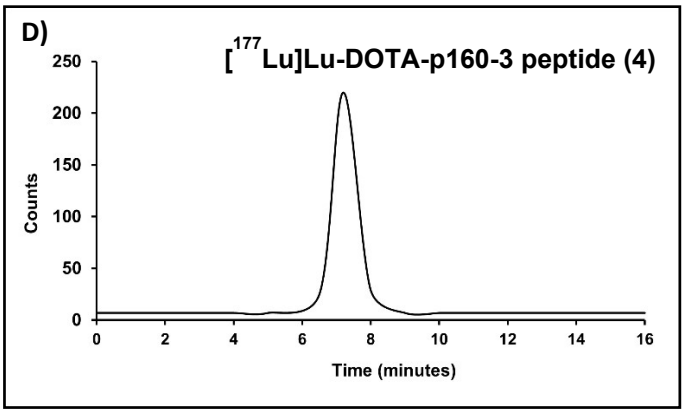

D)
[177Lu]Lu-DOTA-p160-3 peptide (4)
Counts
Time (minutes)

Figure S3. HPLC chromatograms of A) [^{177}Lu]Lu-DOTA-p160 **(1)**; B) [^{177}Lu]Lu-DOTA-p160-1 peptide **(2)**; C) [^{177}Lu]Lu-DOTA-p160-2 **(3)**; [^{177}Lu]Lu-DOTA-p160-3 **(4)**

Proteolytic and Enzymatic Stability

In vitro stability studies in phosphate buffer saline (PBS), human serum and human plasma

In vitro stability of [^{177}Lu]Lu-DOTA-p160 peptide derivatives **(1-4)** was analyzed separately in PBS and in fresh human serum and plasma.[2] To an aliquot (0.2 mL) of the radiolabeled compound, phosphate buffer saline (0.5 mL) was added and the mixture was incubated at 37°C for 1 h in sterile culture tube. Samples were withdrawn from the mixture and analyzed by ITLC and RP-HPLC at 1, 4, 24, 48 and 72 h of incubation period. All results were expressed as mean %SD of three experiments.

[^{177}Lu]Lu-DOTA-p160 peptide derivatives (100 µL) were incubated with human plasma (0.5 mL) at 37℃ for up to 4 h. Following incubation at 1, 4, 24, 48 and 72 h, the plasma proteins were precipitated with acetonitrile/ethanol, and the samples were centrifuged. The supernatant layer was filtered and analyzed by RP-HPLC. All results were expressed as mean %SD of three experiments.

[^{177}Lu]Lu-DOTA-p160 peptide derivatives (100 µL) were incubated with human serum (0.5 mL) at 37°C for up to 4 h. Aliquots (95 µL) were withdrawn at 1, 4, 24, 48 and 72 h of incubation period and poured into 100 µL of methanol to precipitate the proteins followed by cooling on ice for 30 minutes. The samples were centrifuged, and the supernatant was analyzed by RP-HPLC. All results were expressed as mean %SD of three experiments.

In vitro stability studies in enzymatic mixtures

Enzymatic stability of [^{177}Lu]Lu-DOTA-p160 peptide derivatives **(1-4)** was determined by incubation with four enzymes, namely, trypsin, pronase, elastase and pepsin at 37°C.

Proteolysis degradation buffers

The following reaction buffers were used in this study:10 mM PBS at pH 7.5 for trypsin and elastase;10 mM PBS at pH 7.8 for pronase and 10 mM acetic acid±HCl at pH 2.0 for pepsin. DOTA-p160 peptide conjugates **(1-4)** employed in the degradation study were prepared as stock solution of 1 mM in PBS (10 mM, pH 7.5). Peptides were diluted with the selected buffer before incubation with the enzyme.

Proteolytic stability assay

Enzymatic degradation was monitored by incubation of [^{177}Lu]Lu-DOTA-p160 peptide compounds **(1-4)** (900 µL, 300 µM) with the enzyme (100 µL, 50 µM) at 37°C up to 72 h. Aliquots (50 µL) were periodically taken at 0 h to 3 days, 5 µL of 25% acetic acid was added and the degradation was monitored by ITLC and RP-HPLC. All results were expressed as mean %SD of three experiments. As shown in Figure S4 (A-C), DOTA-p160 peptides conjugates **1, 3, 4** were completely resistant to enzymatic degradation under all conditions and no decomposition fragments of

the peptides could be detected. These observations confirmed the superior metabolic stability of the DOTA conjugated p160 peptide derivatives.

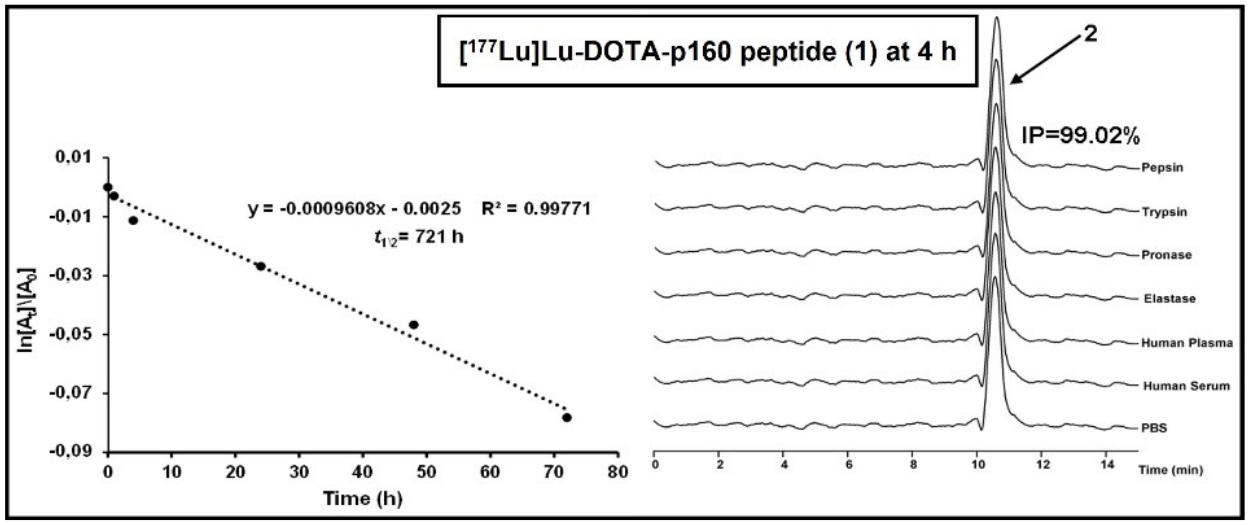


Figure S4 (A). Pseudo first order rate plot of the stability profiles [^{177}Lu]Lu-DOTA-p160 peptide **(1)** in human plasma and RP-HPLC analysis in different incubation media. (IP=Intact peptide percentage).

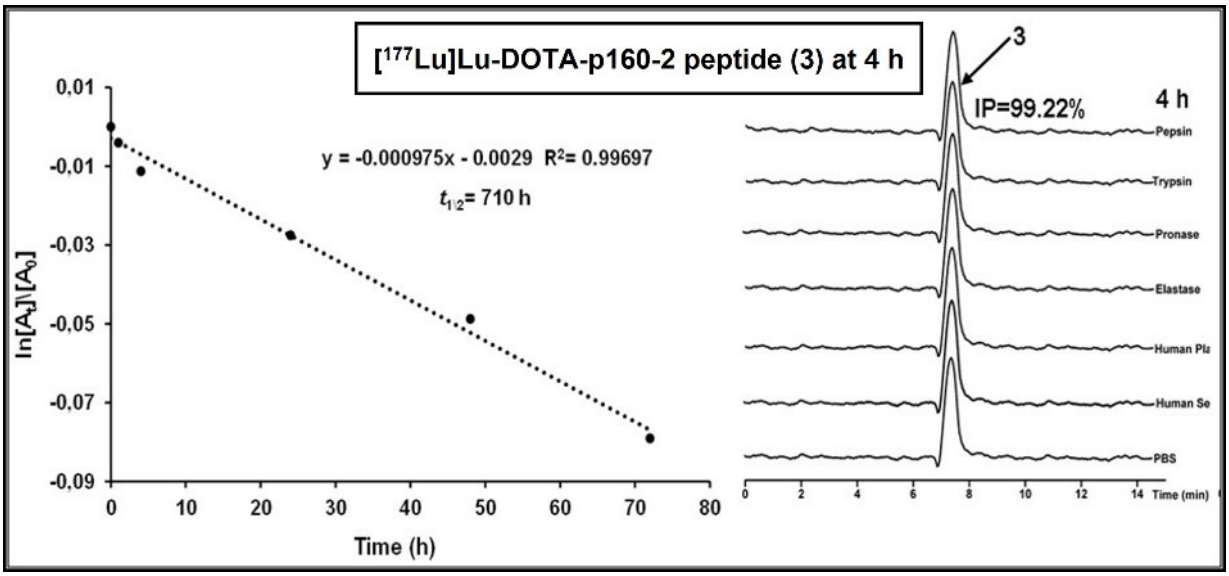


Figure S4 (B). Pseudo first order rate plot of the stability profiles of [^{177}Lu]Lu-DOTA-p160-2 peptide **(3)** in human plasma and RP-HPLC analysis in different incubation media. (IP=Intact peptide percentage).

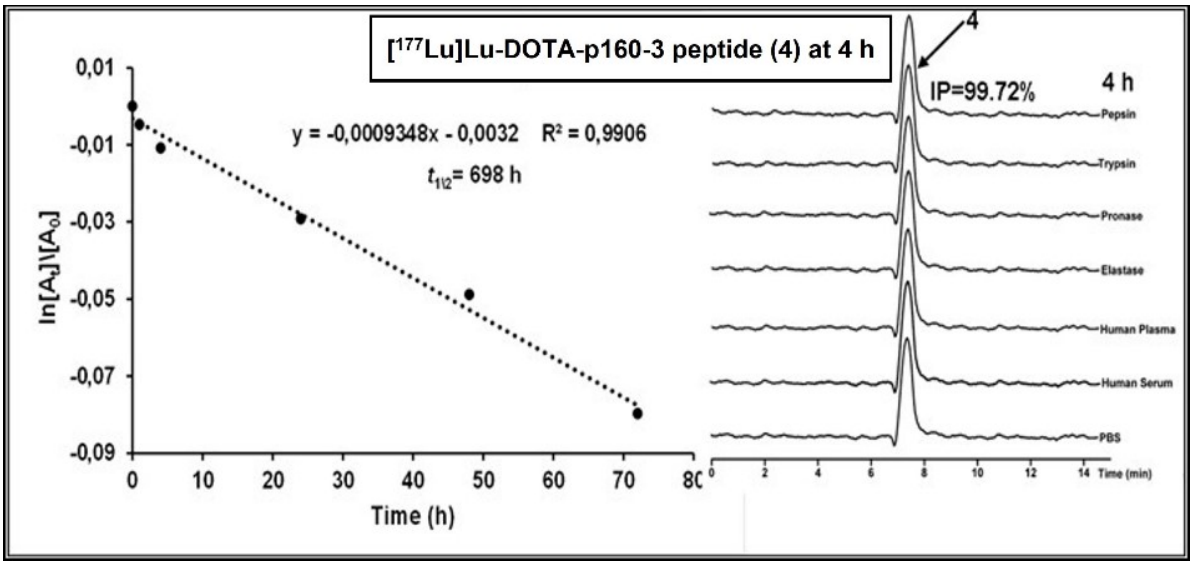


Figure S4 (C). Pseudo first order rate plot of the stability profiles of [^{177}Lu]Lu-DOTA-p160-3 peptide **(4)** in human plasma and RP-HPLC analysis in different incubation media. (IP=Intact peptide percentage).

MTT Assay: in vitro cytotoxicity

The in vitro cytotoxicity of metabolically stable DOTA-p160 peptide derivatives **(1-4)** was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on HEK 293 and HeLa cells. Exponentially growing HeLa and HEK 293 cells were plated in a 96-well microtiter plate at a uniform cell density of 4×10^3 cells/well 24 h before treatment. The DMEM or RPMI growth medium was removed, and cells were treated with the varying concentrations (5–100 μM) of compounds **(1-4)** at time intervals of 24 h and 48 h at 37°C. After treatment, cells were incubated with MTT (0.5 mg/mL) for 4 h at 37 °C. The medium was then carefully removed by aspiration, and the cells were lysed in 200 μL of DMSO to dissolve the formazan crystals. The enzymatic reduction of MTT to formazan crystals was quantified by the optical density measured at 570 nm (ELX800, BioTeK Instruments, USA) with 630 nm as a reference filter. No adverse toxicity on HEK 293 and HeLa cells was observed after 24 h incubation thereby indicating safe and biocompatible nature of the synthesized derivatives for biological use (Figure S5). All assays were done in triplicate and results have been expressed as the mean ± SD.

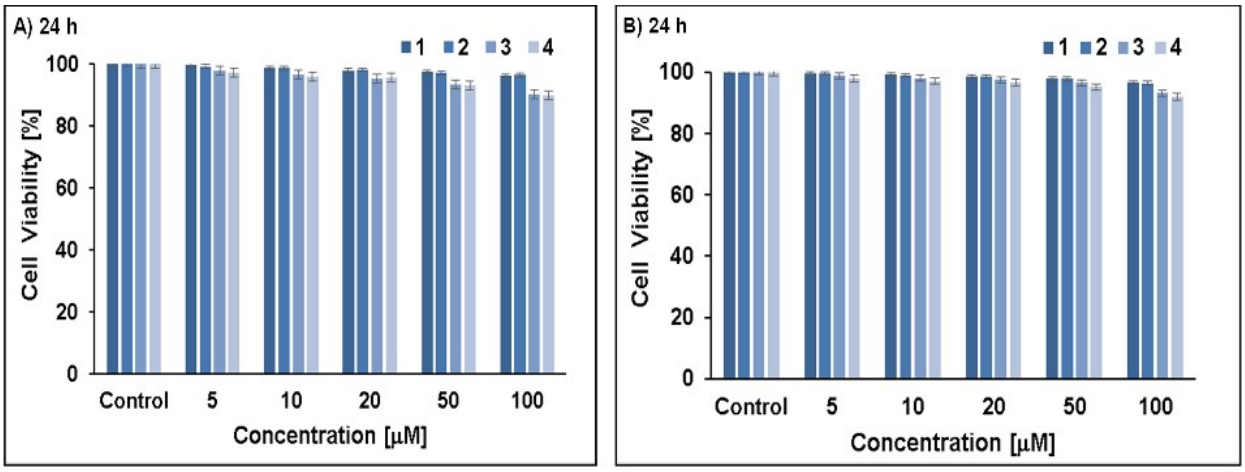


Figure S5. Cytotoxicity of DOTA-p160 **(1)**; DOTA-p160-1 **(2)**; DOTA-p160-2 **(3)** and DOTA-p160-3 **(4)** conjugates monitored in A) HeLa cells (24 h), B) HEK-293 cells (24 h) using MTT assay

Cell Uptake Assays

Cell uptake studies were performed using radiolabeled [^{177}Lu]Lu-DOTA-p160 peptide and derivatives. The specificity of the peptide conjugates to bind to cell surface receptors on tumor cells was examined by receptor binding assays on MCF-7 cell line. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum, 2% glutamine and antibiotics and maintained in humidified atmosphere of 5% CO_2 at 37°C. Confluent monolayers were detached by trypsinization, and cells were suspended in plain DMEM. The cell line cultures were then incubated with various concentrations of [^{177}Lu]Lu-DOTA-p160 peptide and derivatives (0.00001µM – 1µM) in the absence and presence of the 100 folds excess unlabeled p-160 peptide for estimation of total binding and non-specific binding respectively. Specific binding was obtained by subtracting non-specific binding from total binding. At the end of each experiment, the cells were washed with cold PBS and 0.9% saline four times. The cell associated radioactivity was determined by gamma scintillation counting. Scatchard plot analysis was done using EQUILIBRATE software from graph pad.

Statistical analysis

Results are expressed as mean ± S.D. where appropriate.

References

1 D. A. Wellings and E. Atherton, *Methods Enzymol*., 1997, **289**, 44-67.

2 S. Ahmed and K. Kaur, *Chem Biol. Drug Des*., 2009, **73**, 545-52.