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A new class of radiopeptides for PET imaging of neuromedin-B receptor: ^{68}Ga-ranatensin analogs†‡

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The neuromedin B receptor (NMB-R) is a promising target in several human processes, for instance thymic carcinoma, intestinal carcinoids, pruritus, *etc.* NMB-R Positron Emission Tomography (PET) molecular imaging may be of great value. We here report on the development of the first ^{68}Ga-ranatensin analog. The ranatensin derivative (Aib-Gln-Trp-Ala-Val-Gly-His-Phe-Met-$CONH_2$, RV_15) was synthesized and conjugated to the DOTA macrocycle with good yield. This chelator is particularly suitable for ^{68}Ga ($T_{1/2}$ = 67.71 min, β^+ branching = 89.14%) radiolabeling. Radiochemical purity, hydrophilicity, stability, pharmacological properties, inhibitory concentrations (IC_{50}) and biodistribution in normal strain-A mice were investigated. Radiochemical purity of the ^{68}Ga-ranatensin analog was always >97%. The non-radioactive analog, ^{nat}Ga-DOTA–RV_15, was found to be hydrophilic and behaves like an agonist to NMB-R and GRP-R (gastrin-releasing-peptide receptor which is another subtype of bombesin receptor) (EC_{50} values of 5.6 ± 0.1 × 10^{-9} M and 2.1 ± 0.1 × 10^{-9} M, respectively). Moreover, it showed nanomolar binding inhibitory concentrations for human NMB-R (IC_{50} = 3.0 ± 1.1 × 10^{-8} M) and somewhat less for human GRP-R (IC_{50} = 3.2 ± 1.2 × 10^{-7} M) in a competitive binding assay. ^{68}Ga-DOTA–RV_15 is stable in plasma up to 45 minutes. Finally, this ^{68}Ga-ranatensin agonist demonstrated rapid blood and urinary clearances and uptake in the pancreas and kidneys in normal mice. In conclusion, we have developed a novel class of radiopeptide analogue which potentially could be used for NMB-R molecular imaging.

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Introduction

The bombesin receptor (BBN-R) superfamily includes four different subtypes, three of them being expressed in humans: gastrin-releasing-peptide receptor (GRP-R or BB2), neuromedin B receptor (NMB-R or BB1) and BB3 (an orphan receptor).[1–3] The bombesin-like peptides (BLNP) consist of a large number of peptides found in amphibians and mammals, including humans.[4–6] Two BLNP are present in human: gastrin-releasing peptide (GRP)[7] related to bombesin, and neuromedin B (NMB)[8] related to amphibian ranatensin.[5]

GRP and its cognate receptor GRP-R have been extensively studied in various human processes such as prostate cancer and breast cancer[9] and also in itch scratching and nociception.[10]

NMB and its receptor NMB-R have been less investigated but this system is also promising as it is selectively expressed in thymic neuroendocrine tumors and in 46% of intestinal carcinoids[11] and is involved in erectile dysfunction and pruritus.[12]

It is important to mention that among non-CNS tissues, NMB-R is physiologically expressed in the esophagus, smooth muscles of the gastro-intestinal tract, gallbladder and colon.[10,13]

The findings of NMB-R expression in various human processes (and the relatively low expression of NMB-R in human normal tissues) offer opportunities for imaging by designing molecular agents.[9]

To date, no radiolabeled analogs have been yet developed for NMB-R targeting.

Positron Emission Tomography (PET) molecular imaging with ^{68}Ga-DOTA-radiolabeled peptides has demonstrated its clinical utility in patients.[14]

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Hence, we herein report on the development of a ranatensin analog, a new class of radiolabeled peptides. The novel, DOTA-Aib-substituted ranatensin analog (RV_15), suitable for ^{68}Ga Positron Emission Tomography (PET) imaging, was synthesized, radiolabeled and investigated *in vitro*, *ex vivo* and *in vivo*.

Results and discussion

Radiopharmaceutical synthesis

Peptide synthesis and preparation of the reference compound (^{nat}Ga-DOTA–RV_15). Peptide synthesis was carried out using a Rink-Amide AM resin following the standard Fmoc strategy.[15] This peptide was synthesized onto a fully automated peptide synthesizer. Coupling of the resin-bound peptide with the activated ester of DOTA (DOTA-NHS) and cleavage were achieved in DMF at 50 °C with a chemical yield of 55%. The structure of the unlabeled peptide is presented in Fig. 1. (HPLC profile, mass spectrum and elemental analysis are presented in the ESI 1 and 2‡). The reference compound ^{nat}Ga-DOTA–RV_15 was obtained after HPLC purification with a yield of 93%. (HPLC profile and mass spectrum of the reference compound are provided in the ESI 3‡).

Radiolabeling of DOTA–RV_15 with ^{68}Ga

A ^{68}Ga-radiolabeling yield of 27.53 ± 4.20% with a specific activity of 2–4 GBq μmol^{-1} for 30 min after the end of the radiolabeling was obtained. The overall preparation time including quality controls was 60 min. HPLC and TLC analyses indicated that the radiochemical purity of the ^{68}Ga-DOTA–RV_15 preparations always exceeded 97%. The radio-HPLC chromatogram of ^{68}Ga-DOTA–RV_15 is shown in Fig. 2. ^{68}Ga-DOTA–RV_15 has a retention time of 14.7 min and was sterile and pyrogen-free (<2 EU mL^{-1}).

Radiopharmaceutical and pharmacological properties

Octanol/water partition coefficient. To establish the lipophilicity of ^{nat}Ga-DOTA–RV_15, the octanol/water partition coefficient was determined. The compound was hydrophilic with a log *P* value of −3.04 ± 0.33.

***Ex vivo* metabolic stability.** ^{68}Ga-DOTA–RV_15 was tested for stability in human plasma at 37 °C. No significant degradation was observed within 45 minutes. After incubation in human plasma for 2, 30 and 45 minutes, the percentages of intact peptide were 94.2 ± 2.8%, 93.3 ± 1.4% and 93.3 ± 1.1%, respectively (Fig. 3). Radio-HPLC chromatograms are provided in the ESI 4.‡

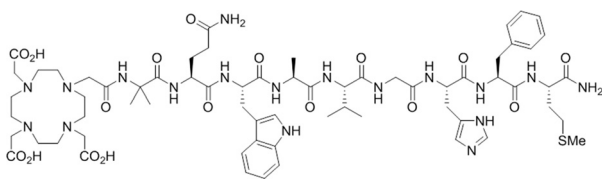


Fig. 1 Structure of unlabeled peptide chelator conjugate DOTA–RV_15.

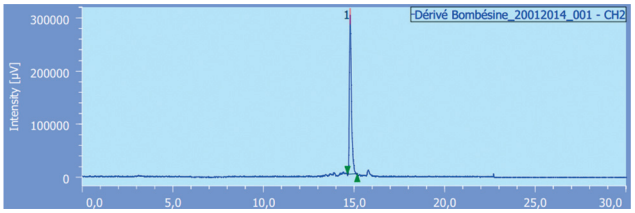


Fig. 2 Representative radio-HPLC chromatogram of ^{68}Ga-DOTA–RV_15, showing high radiochemical purity of the radiolabeled compound.

Calcium imaging. The effects of ^{nat}Ga-DOTA–RV_15 on calcium release were evaluated in NMB-R transfected CHO cells and GRP-R transfected HEK-293 cells. As shown in Fig. 4, ^{nat}Ga-DOTA–RV_15 is able to induce a full control agonist response, even at a low concentration (3×10^{-10} M). Non-linear regression of dose–response curves provided EC_{50} values of $5.6 \pm 0.1 \times 10^{-9}$ M and $2.1 \pm 0.1 \times 10^{-9}$ M for NMB-R and GRP-R, respectively.

Competitive cell binding assay. Bombesin receptor affinities of ^{nat}Ga-DOTA–RV_15 were determined using ^{125}I-Tyr^4-bombesin as radioligand. On human NMB-R, the IC_{50} was $3.0 \pm 1.1 \times 10^{-8}$ M and on human GRP-R, the IC_{50} value was $3.2 \pm 1.2 \times 10^{-7}$ M (Fig. 5).

Normal biodistribution. A biodistribution study was performed in strain-A mice to determine cross reactivity with murine NMB-R and also GRP-R in normal organs and to assess blood and whole-body clearance. ^{68}Ga-DOTA–RV_15 shows rapid blood clearance. Very high uptake was seen in the pancreas (33.0 ± 14.6% ID g^{-1}) and the kidneys (34.4 ± 15.3% ID g^{-1}) at 1 h. Liver uptake was low and low levels of radioactivity were found in other organs (<3% ID g^{-1}). Pre-injection of NMB significantly decreased uptake in the pancreas (6.1 ± 1.0% ID g^{-1}) ($p < 0.05$) and in the kidneys (9.5 ± 4.5% ID g^{-1}) ($p < 0.05$). Similarly, pre-injection of bombesin significantly reduced uptake in the pancreas (9.6 ± 3.5% ID g^{-1}) ($p < 0.05$) and kidneys (7.5 ± 1.5% ID g^{-1}) ($p < 0.05$) at 1 h. A significant decrease in blood uptake is also observed ($p < 0.05$) when blocking with neuromedin B or

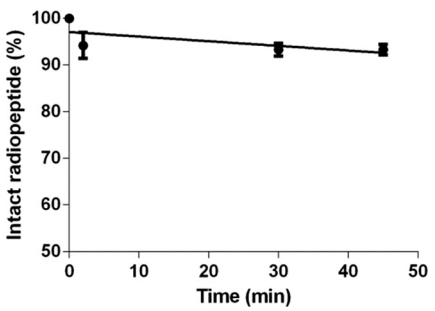
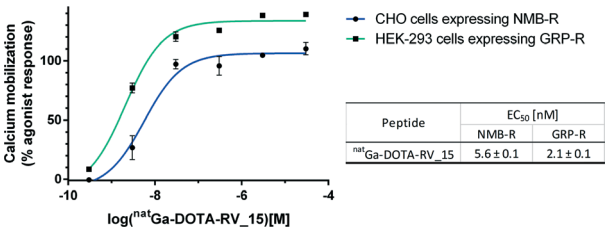
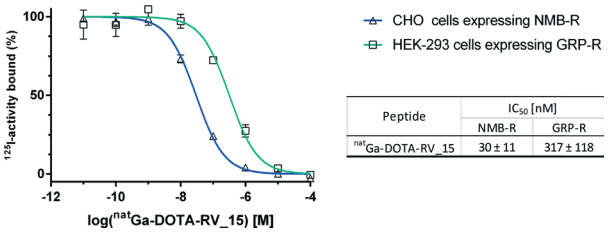


Fig. 3 *Ex vivo* stability of ^{68}Ga-DOTA–RV_15 in human plasma for 0, 2, 30 and 45 minutes at 37 °C. It is considered that the radiopeptide is fully intact at 0 minutes. Values are the mean ± SD of 2 independent experiments.



Peptide	EC_{50} [nM]	
	NMB-R	GRP-R
^{nat}Ga-DOTA-RV_15	5.6 ± 0.1	2.1 ± 0.1

Fig. 4 Pharmacological profiling of ^{nat}Ga-DOTA–RV_15. Calcium imaging experiments with demonstration of calcium release by ^{nat}Ga-DOTA–RV_15. CHO cells expressing hNMB-R and HEK-293 cells expressing hGRP-R were incubated in the presence of increasing concentration of ^{nat}Ga-DOTA–RV_15 (from 3×10^{-10} M to 3×10^{-5} M). Values are the mean ± SD of 2 independent experiments. The values can exceed 100% because a reference agonist has been used for calcium imaging.



Peptide	IC_{50} [nM]	
	NMB-R	GRP-R
^{nat}Ga-DOTA-RV_15	30 ± 11	317 ± 118

Fig. 5 Competition binding curves. CHO cells expressing hNMB-R and HEK-293 cells expressing hGRP-R were incubated in the presence of 0.1 nM ^{125}I-Tyr^4-bombesin (total binding) and an increasing amount of ^{nat}Ga-DOTA–RV_15. Non-specific binding was determined with NMB (1 μM) or GRP (1 μM). The IC_{50} values were 3.0 ± 1.1 × 10^{-8} M for hNMB-R and 3.2 ± 1.2 × 10^{-7} M for hGRP-R.

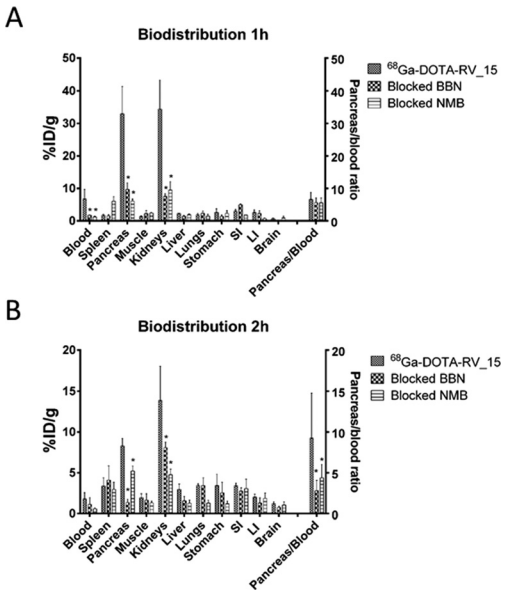


Fig. 6 Normal biodistribution and blocking experiments of ^{68}Ga-DOTA–RV_15 injected in normal mice (n = 3 for each experiment). A: Normal biodistribution at 1 h and NMB-R and GRP-R blocking reveal urinary excretion. B: ^{68}Ga-DOTA–RV_15 normal biodistribution at 2 h and NMB-R and GRP-R blocking revealing urinary excretion and NMB-R and GRP-R mediated uptakes in the pancreas and kidneys. Data are expressed as percentage of injected dose per gram of tissue (% ID g^{-1}). SI is small intestine and LI is large intestine. *Indicates a statistical difference.

bombesin but no statistical differences are demonstrated for the pancreas/blood ratios when neuromedin B or bombesin is pre-infused. At 2 h, no significant decrease is shown by pre-treatment with NMB or BBN in the blood while a significant decrease ($p < 0.05$) is demonstrated by pre-injection of NMB or BBN in the pancreas and kidneys. Pre-infusion of neuromedin B and bombesin also led to a significant decrease of the pancreas/blood ratio at 2 h. The radioactivity wash-out was fast: the radioactivity concentration in the pancreas decreased more than 3 fold between 1 h and 2 h (Fig. 6).

In this study, we synthesized the DOTA-conjugated ranatensin analog Aib-Gln-Trp-Ala-Gly-Val-His-Phe-Met-$CONH_2$ (RV_15). The modified peptide bears the ranatensin Ran[7–14]-NH_2 sequence. Therefore, this ranatensin analog is the first of a new class of peptide analog which is unused in the imaging field. α-Aminoisobutyric acid (Aib) was used both as a hydrophilic linker and a stabilizer of the structure of the DOTA conjugated peptide. Aib is a naturally occurring amino acid produced by microbial sources that belongs to the class of α,α-disubstituted amino acids but is non proteinogenic in humans. Adding to the overall hydrophilicity, Aib is also expected to provide favorable pharmacokinetic properties such as a stronger resistance to proteases *in vivo*. Since it has been reported that BBN adopts a helical structure in the region [Asn^6-Met^{14}],[16] we incorporated the conformationally restricted Aib-amino acid, in the ranatensin sequence, aiming that it would stabilize the conformation and possibly enhance its activity. This finding is in line with a previous study showing that a rigid linker improves tumor uptake and retention.[17]

The DOTA chelate will be suitable for labelling with many radionuclides, such as ^{68}Ga, ^{64}Cu or ^{44}Sc for PET imaging or ^{177}Lu, ^{67}Cu or ^{161}Tb for peptide receptor radionuclide therapy.[13] ^{68}Ga is attractive due to its easy production through a ^{68}Ge/^{68}Ga generator and its short half-life suitable for pharmacokinetics of many peptides.[18]

The peptide was therefore radiolabeled with ^{68}Ga, with a moderate labeling yield and specific radioactivity (due to the age of the generator used when performing experiments: 13 months), which permitted the use of the radiolabeled conjugate for both *in vitro* and *in vivo* experiments. No free ^{68}Ga was detected after purification. The retention time of DOTA–RV_15 increases when Ga^{3+} ions are added, most probably due to the change in geometry and overall charge of the DOTA chelator upon complexation.

The log P value was consistent with the expected hydrophilicity. The data reveals a hydrophilic compound, pointing out that it should show rapid blood clearance, preferential renal excretion and low hepatobiliary excretion. For instance, it has been recently demonstrated that a marked hydrophilicity confers advantageous biodistribution of BBN analogs.[19]

Another important prerequisite for a radiopeptide designed for tumor targeting is its high metabolic stability in human plasma since it is mandatory for the radiolabeled

peptide to reach its target intact. Moreover, a high tissue-to-background ratio could be also expected if the concentrations of radiometabolites are as low as possible. Our results are in agreement with the expected low enzymatic degradation by plasma proteases provided by the incorporation of Aib in the ranatensin sequence.

The binding profile of ^{nat}Ga-DOTA–RV_15 to the bombesin receptors was assessed, showing good to moderate binding affinities to NMB-R (IC_{50} = 3.0 ± 1.1 × 10^{-8} M) and GRP-R (IC_{50} = 3.2 ± 1.2 × 10^{-7} M). ^{nat}Ga-DOTA–RV_15 shows a 10-fold higher affinity for NMB-R which sets our compound apart.

In this study, cell calcium imaging experiments demonstrated that ^{nat}Ga-DOTA–RV_15 is an NMB-R agonist as it induced a full neuromedin B response with good EC_{50}. With respect to GRP-R, ^{nat}Ga-DOTA–RV_15 behaves also as an agonist with a similar EC_{50} value although the GRP-R affinity is ten times lower than the NMB-R affinity (Fig. 4). These data suggested that our ranatensin analog is an NMB-R agonist and also a GRP-R agonist.

One hour after administration in normal mice, ^{68}Ga-DOTA–RV_15 underwent urinary excretion and showed high uptake in the pancreas and kidneys. Pre-injection of NMB or BBN significantly decreased uptake in the pancreas and kidneys and also in the blood. Hence, pancreas and kidney blocking performed at 1 h appears to be mostly driven by blood uptake (pancreas/blood and kidneys/blood ratios are not significantly decreased by pre-injection of NMB or BBN, data not shown for kidneys). ^{68}Ga-DOTA–RV_15 biodistribution demonstrated a similar pattern to the profile at 2 h but not to that at 1 h, with the exception that there is no significant decrease in blood when blocking with either NMB or BBN was performed even if a trend toward a reduced blood activity may not be excluded. A significant decrease in ^{68}Ga-DOTA–RV_15 uptake was seen in the pancreas and kidneys. This finding suggests that the uptake in the pancreas and kidneys was NMB-R/GRP-R mediated which is in accordance with the known expression of bombesin receptors in mice pancreas[19] (the contribution of reduced activity in blood could not be totally excluded, even if not statistically apparent). Uptake in the other organs remains low (<3% ID g^{-1}) at 1 h and 2 h post injection and is not significantly decreased by the blocking effect of NMB or BBN. Taken together, these biodistribution data suggest that a good tumor/non-tumor ratio could be obtained, preferentially 2 h after injection (Fig. 6).

A clear limitation of our study to assess the potential of ^{68}Ga-DOTA–RV_15 is the absence of imaging studies due to the lack of rigorous data in the literature regarding human cell lines expressing only NMB-R (not co-expressing other bombesin receptors). The lack of studies conducted with highly validated antibodies or radiolabeled agents targeting NMB-R makes the choice of relevant human cell lines for imaging tricky. Therefore, we were not able to provide μPET imaging to document specific NMB-R targeting.

No analogs have yet been designed for specific NMB-R targeting. Our study was an attempt to target NMB-R with a radiolabeled peptide and further optimization could lead to increased NMB-R affinity and selectivity. Nevertheless, in processes that express NMB-R, ranatensin analogs might offer a novel imaging and/or therapeutic modality. The discovery of NMB-R in various human pathophysiological situations will stimulate the development of agents for NMB-R targeting.

Experimental

A widely used approach for the development of a peptide-based agent is the designing of conformationally constrained bioactive peptide derivatives. The α-aminoisobutyric acid (Aib) in the peptide sequence restricts the rotation of the θ, ψ angles within the molecule and induces conformational constraint in the peptide backbone, thereby stabilizing a desired peptide conformation. Aib has been shown to induce a ß-turn in small peptides and a helical conformation in larger peptides.[20] Aib has been incorporated into the peptide sequence to design analogs of different bioactive peptides *i.e.* chemotactic peptide,[21,22] somatostatin,[23] GnRH,[24] parathyroid hormone,[25] and bombesin.[26]

Materials

All chemicals, unless otherwise stated, were used without further purification. Peptide synthesis was carried out using a Rink-Amide AM resin (200–400 mesh, 0.71 mmol g^{-1}) from Merck. All protected amino acids (Fmoc-Aib-OH, Fmoc-Gln(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-His(Trt)-OH, Fmoc-Phe-OH and Fmoc-Met-OH) were L-amino acid residues. These L-amino acid residues and HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate) were purchased from Merck. DIEA (*N*,*N*′-diisopropylethylamine) and DMF used during the coupling reaction were purchased from Sigma-Aldrich. Piperine, HOBT (1-hydroxybenzotriazole), TFA (trifluoroacetic acid), triisopropylsilane, and phenol used during the deprotection and cleavage steps were purchased from Sigma-Aldrich. DOTA-NHS ester (2,2′,2″-(10-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)-triacetic acid) was purchased from CheMatech. $^{nat}Ga(NO_3)_3 \cdot 9H_2O$ used to synthesize the reference peptide was purchased from Strem chemicals. ^{68}Ga ($T_{1/2}$ = 67.71 min, β^+ = 89.14%) was obtained from a ^{68}Ge/^{68}Ga generator (IASON®, nominal activity 1480 MBq, Cyclotron Co., Ltd, Obninsk, Russia) as previously described.[27] The 5 M sodium chloride solution, sodium acetate buffer (pH 4.6) and phosphate buffered saline used for the radiolabeling were purchased from Sigma-Aldrich. Ultrapure 30% HCl, deionized water and ethanol were purchased from Merck.

High-performance liquid chromatography (HPLC) analyses were performed using a Phenomenex Luna C_{18} (250 × 4.6 mm, 5 μm, 1 mL min^{-1}) and the indicated conditions. The analytical HPLC system used was a JASCO system with ChromNAV software, a PU-2089 Plus quaternary gradient pump, a MD-2018 Plus photodiode array detector and a Raytest Gabi Star detector. Semi-preparative HPLC purifications were carried out using a semi-preparative Phenomenex Luna C_{18} column (250 × 10 mm, 5 μm, 5 mL min^{-1}) and the

indicated conditions. The semi-preparative HPLC system used was a JASCO system equipped with two PU-2086 pumps and a UV-2075 multi-wavelength detector.

Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) was performed on a Voyager mass spectrometer (Applied Biosystems) equipped with a pulsed N_2 laser (337 nm) and a time-delayed extracted ion source.

Chemical and radiopharmaceutical syntheses

Solid phase peptide synthesis. The ranatensin derivative (Aib-Gln-Trp-Ala-Val-Gly-His-Phe-Met-$CONH_2$ (RV_15)) was synthesized by solid-phase synthesis onto a Liberty 1 (CEM) microwave peptide synthesizer using a Rink-Amide AM resin (200–400 mesh), applying the standard 9-fluorenylmethyloxycarbonyl (Fmoc) strategy.[15] The resin was allowed to expand for 30 min in DMF (10 mL) before synthesis. The peptide synthesis was performed at a 0.25 mmol scale starting with 352 mg of the resin.

Fmoc-deprotection (general procedure). The resin-bound Fmoc peptide was treated twice with 10 mL of 20% piperidine w/ 0.1 M HOBT in DMF for 180 seconds at 70 °C (45 Watts). Then the resin was washed with DMF (3 × 10 mL).

HBTU/DIEA coupling (general procedure). Fmoc-amino acid (5 equiv., 5 mL) in DMF was added to the reactor. Then, HBTU (5 equiv., 2 mL) followed by DIEA (5 equiv., 1 mL) in DMF were successively added in the reactor and the reaction mixture was heated at 70 °C (45 watts) for 300 seconds. Then the resin was washed with DMF (3 × 10 mL).

HBTU/DIEA coupling (His coupling). Fmoc-His-OH (5 equiv., 5 mL) in DMF was added to the reactor. Then, HBTU (5 equiv., 2 mL) and DIEA (5 equiv., 1 mL) in DMF were successively added in the reactor and the reaction mixture was heated at 50 °C without microwaves for 120 seconds and then under microwaves at 50 °C (30 Watts) for 240 seconds. The resin then was washed with DMF (3 × 10 mL).

DOTA conjugation and final cleavage/deprotection. The resin-bound, side chain protected peptide was suspended in dry DMF (20 mL). The DOTA-NHS ester (476 mg, 0.625 mmol, 2.5 equiv.) was added to the reaction mixture followed by triethylamine (348 μL, 2.5 mmol, 10 equiv.). The reaction mixture was heated at 50 °C for 2 days and then the resin was filtered and washed with DMF. Subsequent treatment of the resin with 2 mL of TFA/water/triisopropylsilane/phenol (88 : 5 : 5 : 2) afforded the fully deprotected DOTA-peptide, which was precipitated and washed with cold diethyl ether. The crude product was dried *in vacuo*, purified by semi-preparative HPLC. DOTA–RV_15 was eluted with 90% A (0.1% TFA in water) and 10% B (0.1% TFA in acetonitrile) for 2 min and 90% B gradient for 25 min. The purified fractions were collected and then lyophilized to afford DOTA–RV_15(198 mg, 55%). The product was analyzed by MALDI-TOF-MS: m/z [M + H^+] calculated: 1445.7 found: 1445.6, analytical HPLC (gradient 95/5 to 5/95 (H_2O 0.1% TFA/ACN 0.1% TFA) in 45 min, flow 1 mL min^{-1}, wavelength 280 nm). The retention time is 17.3 min.

Labeling of DOTA–RV_15 with ^{nat}Ga (reference compound). DOTA–RV_15 (15.5 mg, 10 μmol) was dissolved in 1 mL of acetate buffer (pH 4.5). $^{nat}Ga(NO_3)_3{\cdot}9H_2O$ (5 mg, 12 μmol, 1.2 equiv.) was added and the reaction mixture was heated at 80 °C for 20 min under microwave in a Biotage Initiator Classic. The crude mixture was purified similarly to the procedure described above for DOTA–RV_15 to give ^{nat}Ga-DOTA–RV_15 (14 mg, 93%) after lyophilisation. MALDI-TOF-MS: m/z [M + H^+] calcd.: 1511.6 found: 1511.4. Analytical HPLC (same conditions as the precursor) showed that the retention time of the reference compound is 17.7 min.

Radiolabeling of DOTA–RV_15 with ^{68}Ga. A fully automatic, radiopharmaceutical synthesis device (GE FastLab, GE Healthcare, GEMS Benelux, Belgium) was used for all elution and radiolabeling steps as previously described.[27] ^{68}Ga was eluted from the ^{68}Ge/^{68}Ga generator with 0.1 M HCl (starting activity ~ 400 MBq) and purified/concentrated by solid phase extraction onto a Chromafix 30 PS-HCO_3 cartridge (Macherey-Nagel). Concentrated ^{68}Ga (200 μL) was added to the reactor vial containing DOTA–RV_15 (75 μg) in 1.5 mL of acetate buffer (pH 4.6). The reaction mixture was incubated at 95 °C for 5 min under microwaves. The crude ^{68}Ga-DOTA–RV_15 was purified using a C_{18} cartridge (Sep-Pak Light C_{18}, WAT023501 Waters). The purified product was eluted with 1 mL of absolute ethanol and formulated to 10 mL with PBS, water, 0.9% sodium chloride (pH 7.4) and 0.3 mM ascorbic acid. The resulting solution was passed through a 0.22 μm filter into a sterile injection vial to determine the radiochemical purity (RCP), radionuclidic identification (RNI) and pH. Specific radioactivity (SRA) was determined by the ratio between the ^{68}Ga-radioactivity, measured in the calibrated ionization chamber,[28] and the amount of ^{68}Ga-DOTA–RV_15. The amount of the radiolabeled product was calculated by HPLC analysis by using the calibration curve of the reference ^{nat}Ga-DOTA–RV_15. The RCP of ^{68}Ga-DOTA–RV_15 was determined by HPLC and confirmed by TLC analysis. The applied gradient was 90% A (0.1% TFA in water) and 10% B (acetonitrile) for 5 min, 90% B at 26 min, and 10% B at 28 min. For TLC analysis, the RCP was determined by using ITLC/SG strips (8 cm × 1 cm) and ammonium acetate (77 g L^{-1}) : methanol (1 : 1 v/v) (free ^{68}Ga $R_f \leq 0.1$ and bound ^{68}Ga $R_f \geq 0.8$) as running buffer. The RNI of ^{68}Ga-DOTA–RV_15 was determined by γ-spectrometry (Raytest-Gabi). Samples were also tested for endotoxins (Charles Rivers) and sterility.

Pharmacological characterisation

Octanol/water partition coefficient (Log *P*). To an Eppendorf tube filled with 0.5 mL of the ^{nat}Ga-DOTA–RV_15 previously dissolved in water, 0.5 mL of octanol was added. The tube was vigorously stirred by a vortex for 2 min at room temperature and the 2 layers were separated by centrifugation (4000 rpm, 3 min). Samples of 20 μL were taken from each layer and the amount of ^{nat}Ga-DOTA–RV_15 in each layer was determined by analytical HPLC. Then the log *P* values (pH 7.4) were calculated.

***Ex vivo* metabolic stability.** The stability of ^{68}Ga-DOTA–RV_15 was investigated in fresh human plasma at various incubation times (0, 2, 30 and 45 min) at 37 °C as previously described.[29] PBS was used as a control. After incubation, the serum was passed through a Sep-Pak C_{18} cartridge, washed with 0.5 mL of PBS buffer, and then eluted with 0.5 mL of ACN containing 0.1% TFA. The PBS control was diluted with 0.5 mL of acetonitrile. The elution fractions and the PBS control were analyzed by radio-HPLC and all fractions were checked for activity. The data are expressed as percentage of initial activity and were corrected for ^{68}Ga-decay.

Calcium imaging. Calcium imaging experiments have been performed as previously described on a transfected cell line expressing GRP-R or NMB-R.[30] CHO cells expressing hNMB-R and HEK-293 cells expressing hGRP-R were incubated in the presence of increasing concentration of ^{nat}Ga-DOTA–RV_15 (from 3×10^{-10} M to 3×10^{-5} M). Control response has been determined by using NMB (10 nM) or GRP (3 nM).

Competitive cell binding assay. Binding assay has been performed as previously described.[31,32] Briefly, the IC_{50} values of ^{nat}Ga-DOTA–RV_15 were determined by using human recombinant CHO cells transfected with human NMB-R and human recombinant HEK-293 cells transfected with human GRP-R. The radioligand used was ^{125}I-Tyr^4-bombesin (0.1 nM; $K_{d,NMBR}$ = 0.1 nM; $K_{d,GRPR}$ = 0.04 nM) and non-specific binding was evaluated using neuromedin B (1 μM) and GRP (1 μM).

Normal biodistribution. All animal experiments/protocols were performed in accordance with the guidelines of INMAS animal ethics committee (Regn. no: 8/GO/a/99/CPCSEA). The animals were sacrificed using the cervical dislocation method and also all animal experiments were performed in accordance with the relevant laws (Animals ACT 1986) and the guidelines of INMAS animal ethics committee (no. INM/DASQA/1AEC/09/15). The institutional animal ethics committee approved the experiments.

To investigate the normal biodistribution of ^{68}Ga-DOTA–RV_15, strain-A mice were studied. Groups of three mice per data points were used.

The labeled compound ^{68}Ga-DOTA–RV_15 was diluted at a concentration of 3.3 MBq per 100 μL before being distributed into syringes where radioactivity was measured before and after injection. A volume of 0.1 mL (3.7 MBq) of solution was injected into a lateral tail vein of each animal. The study of ^{68}Ga-DOTA–RV_15 was performed in three groups of six mice and sacrificed at 1 h and 2 h after administration. The first group was injected with 1.8 nmol of ^{68}Ga-DOTA–RV_15. To test the *in vivo* binding specificity, two other groups of animals were injected with 62 nmol of bombesin (Bachem®) or 88 nmol of neuromedin B (Bachem®) 15 minutes before injection of the radiolabeled peptide. Blood was collected by cardiac puncture.

Blood and other tissues (liver, kidney, pancreas, lungs, heart, bone, intestines and muscles) were removed from each animal and weighed before the radioactivity was measured in a γ-counter and compared with the standard. The results were expressed as percentage of injected dose per gram (% ID g^{-1}) of tissue.

Statistical analyses

All mean values are given ± SD. Non-linear regression of curves and two-way ANOVA with Tukey's multiple comparisons test were performed using GraphPad Prism (GraphPad Software, Inc. Version 6.02). The level of significance was set at $p < 0.05$.

Conclusions

To the best of our knowledge, we have synthesised and radiolabeled with ^{68}Ga the first Aib-ranatensin analog. ^{68}Ga-DOTA–RV_15 is hydrophilic *in vitro* and metabolically stable *ex vivo*. ^{68}Ga-DOTA–RV_15 exhibits agonistic properties with respect to NMB-R and GRP-R. Gallium-labeled DOTA–RV_15 binds with high affinity to NMB-R and moderate affinity to GRP-R. In normal mice, uptake is shown in the pancreas and the kidneys. Finally, ^{68}Ga-DOTA–RV_15 underwent urinary excretion. Ranatensin analogs might therefore be suitable for PET molecular imaging of NMB-R-expressing cells. Further optimization of our compound is needed to increase NMB-R affinity and selectivity but we are convinced that NMB-R targeting has a radiant future.

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