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Design, Synthesis, and Biological Evaluation of the First Radiometalated Neurotensin Analogue Targeting Neurotensin Receptor 2

Sacha Bodin, Santo Previti, Emmanuelle Jestin, Delphine Vimont, Imade Ait-Arsa, Frédéric Lamare, Emmanuelle Rémond, Elif Hindié, Florine Cavelier,^{*,#} and Clément Morgat^{*,#}



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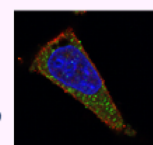
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Supporting Information

ABSTRACT: Neurotensin receptor 2 (NTS₂) is a well-known mediator of central opioid-independent analgesia. Seminal studies have highlighted NTS₂ overexpression in a variety of tumors including prostate cancer, pancreas adenocarcinoma, and breast cancer. Herein, we describe the first radiometalated neurotensin analogue targeting NTS₂. JMV 7488 (DOTA-(βAla)₂-Lys-Lys-Pro-(D)Trp-Ile-TMSAla-OH) was prepared using solid-phase peptide synthesis, then purified, radiolabeled with ⁶⁸Ga and ¹¹¹In, and investigated *in vitro* on HT-29 cells and MCF-7 cells, respectively, and *in vivo* on HT-29 xenografts. [⁶⁸Ga]Ga-JMV 7488 and [¹¹¹In]In-JMV 7488 were quite hydrophilic (logD_{7.4} = -3.1 ± 0.2 and -2.7 ± 0.2, respectively, *p* < 0.0001). Saturation binding studies showed good affinity toward NTS₂ (K_D = 38 ± 17 nM for [⁶⁸Ga]Ga-JMV 7488 on HT-29 and 36 ± 10 nM on MCF-7 cells; K_D = 36 ± 4 nM for [¹¹¹In]In-JMV 7488 on HT-29 and 46 ± 1 nM on MCF-7 cells) and good selectivity (no NTS₁ binding up to 500 nM). On cell-based evaluation, [⁶⁸Ga]Ga-JMV 7488 and [¹¹¹In]In-JMV 7488 showed high and fast NTS₂-mediated internalization of 24 ± 5 and 25 ± 11% at 1 h for [¹¹¹In]In-JMV 7488, respectively, along with low NTS₂-membrane binding (<8%). Efflux was as high as 66 ± 9% at 45 min for [⁶⁸Ga]Ga-JMV 7488 on HT-29 and increased for [¹¹¹In]In-JMV 7488 up to 73 ± 16% on HT-29 and 78 ± 9% on MCF-7 cells at 2 h. Maximum intracellular calcium mobilization of JMV 7488 was 91 ± 11% to that of levocabastine, a known NTS₂ agonist on HT-29 cells demonstrating the agonist behavior of JMV 7488. In nude mice bearing HT-29 xenograft, [⁶⁸Ga]Ga-JMV 7488 showed a moderate but promising significant tumor uptake in biodistribution studies that competes well with other nonmetalated radiotracers targeting NTS₂. Significant uptake was also depicted in lungs. Interestingly, mice prostate also demonstrated [⁶⁸Ga]Ga-JMV 7488 uptake although the mechanism was not NTS₂-mediated.

[M⁺]M-DOTA-(βAla)₂-Lys-Lys-Pro-(D)Trp-Ile-TMSAla-OH



Targeting NTS₂-expressing tumors for PET imaging and therapy

INTRODUCTION

G-protein coupled receptors (GPCRs) represent numerous targets for drug development,¹ particularly in nuclear medicine. Recent advances in nuclear oncology include the use of [⁶⁸Ga]Ga-DOTATOC and [¹⁷⁷Lu]Lu-DOTATATE for diagnosis and peptide receptor radionuclide therapy (PRRT) of digestive neuroendocrine tumors overexpressing the somatostatin receptor subtype 2 (SST₂), which is part of the class A GPCR family. Among this same family, neurotensin (NT) receptors represent also promising targets for imaging and therapy. Neurotensin is a 13 amino acid neuropeptide,² which binds to three receptors, two GPCRs, namely, NTS₁ and NTS₂, and the single transmembrane domain NTS₃/sortilin receptor. NTS₁ and NTS₂ are widely expressed in the central nervous system (CNS) where they are involved in opioid independent analgesia, hypothermia, and psychiatric effects,³ but they also exhibit peripheral actions.^{4,5}

Numerous NTS₁ targeting ligands carrying the pharmacophore NT(8-13) hexapeptide sequence (H-Arg-Arg-Pro-Tyr-Ile-Leu-OH)^{6,7} have been developed and studied in different types of peripheral tumors, such as colorectal cancer, prostate adenocarcinoma, breast cancer, or pancreatic cancer.^{8–12} Contrarily, NTS₂ targeting peptides have been studied for

their central opioid-independent analgesic potency.^{13–17} Whereas very few analogues have been developed for imaging of peripheral tumors,¹⁸ NTS₂'s role in tumor development remains unclear and requires more investigations. Indeed, in prostate cancer, NTS₂ mRNA expression was found only in malignant well-differentiated (androgen receptors +) cell-lines.¹⁹ A recent study also demonstrated its involvement in apoptosis resistance in B-cell chronic lymphocytic leukemia.²⁰

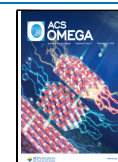
Only few studies have investigated the expression of NTS₂ in normal organs. In healthy tissues, NTS₂ has been found to be expressed in lungs.²¹

Herein, we report the pharmacological and biological properties of JMV 7488, a new silylated NTS₂-targeting hexapeptide radiolabeled with gallium-68 and indium-111. This ligand is derived from the N-terminal elongation of JMV

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5504, a modified NT(8-13) analogue, which has high affinity and selectivity toward the NTS₂ receptor.²²

MATERIALS AND METHODS

Chemistry, radiolabeling procedure, determination of the octanol/water partition coefficient, cell culture, western blot, confocal imaging, and *blood plasma* stability methods are fully presented in the [Supporting Information](#).

In Vitro Studies. HT-29 and MCF-7 cells were seeded into 24-wells plates at the density of 2×10^5 cells per well, the day before the experiment. The plates were incubated at 37 °C and 5% CO₂. All the binding and internalization experiments were carried out in quadruplicate and efflux studies in octuplicate.

Saturation Binding Studies. Plates were stored at 4 °C for 30 min in order to reduce cell processing and then incubated for 2 h at 37 °C with 250 μL of complete medium containing the radiolabeled peptide at increasing concentrations (0.1, 1, 10, 100, 250, and 500 nM) with and without saturation ligands (neurotensin, a specific agonist of NTS₁ or levocabastine, a specific agonist of NTS₂, final concentration of 1 μM). After incubation, cells were rinsed twice with ice cold DPBS (250 μL, 14190-144, Gibco). Then, cells were lysed with NaOH 1 M (750 μL) to collect the bound fraction. Radioactivity of each fraction was determined in a gamma-counter (Wizard2, PerkinElmer, USA). Affinity (K_D) was determined by nonlinear regression using Prism 6.01 software (GraphPad Software Inc., USA). Experiments were performed three times in quadruplicates.

Cell-Associated Radioactivity and Internalization Studies. A total of 1 MBq of [⁶⁸Ga]Ga-JMV 7488 or 50 kBq of [¹¹¹In]In-JMV 7488 in 250 μL of complete medium with and without 1 μM of saturation ligands was added in each well. Plates were incubated for 10, 30, or 60 min (plus 120 and 240 min for ¹¹¹In radiolabeled JMV 7488) at 37 °C. Three minutes before the selected time point, plates were stored at 4 °C to stop the internalization process. Media were removed, and wells were rinsed three times with ice cold DPBS (250 μL). A total of 250 μL of sodium acetate (20 mM, pH 5) was added in each well twice and then collected in tubes after 5 min incubation. Then, cells were lysed with NaOH 1 M (750 μL) to collect the bound fraction. Radioactivity of each fraction was determined in a gamma-counter. Cell-associated radioactivity results are expressed as percentage of specific binding. Experiments were performed four times in quadruplicates.

Efflux Studies. A total of 1 MBq of [⁶⁸Ga]Ga-JMV 7488 or 50 kBq [¹¹¹In]In-JMV 7488 in 250 μL of complete medium was added in each well. Plates were incubated 30 min at 37 °C. Media were removed, and cells were rinsed with ice cold DPBS (250 μL). A total of 250 μL of sodium acetate (20 mM, pH 5) was added in each well and removed after 5 min of incubation. Cells were rinsed again with ice cold DPBS (250 μL). Then, 250 μL of medium were added in each well and plates were incubated for 5, 15, 30, or 45 min (or 5, 15, 60, and 120 min for ¹¹¹In radiolabeled JMV 7488) at 37 °C. Media were collected in tubes, and each well was rinsed twice with ice cold DPBS (250 μL). Then, cells were lysed with NaOH 1 M (750 μL) to collect the bound fraction. Radioactivity of each fraction was determined in a gamma-counter. Results are expressed as percentage of total binding. Experiments were performed three times in octuplicate.

Calcium Imaging Assay. HT-29 cells were cultured as previously described. The day before the experimentation, cells

were grown on 15 mm Thermanox glass slides (ThermoFischer, USA) in 12-wells plates at the density of 5×10^5 cells in 500 μL of complete culture medium. Night incubation at 37 °C and 5% CO₂ allows cell adherence on the slides. Glass slides were individually loaded for 30 min in the dark with a 500 μL loading solution containing 100 μM fluo-8 AM (ATT Bioquest, USA), 0.4% (w/v) pluronic acid (AAT Bioquest, USA), and 2.5 mM probenecid (Invitrogen, ThermoFischer, USA) into RPMI medium. Slides were then placed in a recording chamber perfused with buffer solution (containing (in mM): 130 NaCl, 3 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 0.58 NaH₂PO₄, 25 NaHCO₃, and 10 glucose) equilibrated with 95% O₂/5% CO₂, adjusted to pH 7.4 at room temperature. A 10 min delay was respected to wash out extracellular fluo-8 AM and enable fluo-8AM intracellular hydrolysis. Increasing concentrations (10^{-11} to 10^{-5} M) of JMV 7488 were added *via* a rapid perfusion system. Positive control experiments were performed by adding similar concentrations of levocabastine. Imaging of intracellular Ca²⁺ changes was performed following excitation of cells at 490 nm with analysis of fluorescence emission at 514 nm using an Eclipse E600FN microscope (Nikon, Japan) coupled with an Infinity 3 detector (Lumenera, Canada). Changes in fluorescence (F_T) were calculated for each cell relative to the averaged baseline fluorescence before stimulation (F_{REST}) and expressed as $\Delta F/F$ (%) = $[(F_T - F_{REST})/F_{REST}] \times 100$. The calcium mobilization assay was performed twice in duplicate, and 20 cells were manually analyzed.

In Vivo Studies. Animal experiments have been approved and permitted by the Ethics committee n°114 (reference APAFIS#26960-2020061008304944 v4) and were conducted according to European and national guidelines. All the animals were maintained at a constant temperature (24 ± 1 °C) and a hygrometry between 55 and 65% under a 12 h light/dark cycle and permitted free access to food and water.

HT-29 Xenograft Model. Eight weeks old female Balb-c nude mice have been purchased from JANVIER LABS, and quarantined for 2 weeks. Animals are fed *ad libitum* and maintained in a HEPA-filtered environment with cages, food, and bedding sterilized by autoclaving.

Under anesthesia with isoflurane (2% in air mixture), Balb-c nude mice were injected subcutaneously in the right flank with 1×10^6 HT-29 cells suspended in 100 μL of 50:50 Matrigel/collagen I. Tumor growth was monitored externally using calipers. Biodistribution and PET/CT imaging studies were performed in mice when tumor volume reached approximately 150 mm³.

Micro-PET/CT Imaging. The preclinical imaging studies were performed on a multimodality imaging system (Triumph II, PET/SPECT/CT, Trifoil Imaging, Chatsworth, USA). Mice were anesthetized with isoflurane (3%) and placed under the PET modality on a temperature-controlled bed. Injections of the radiotracers were proceeded in the caudal lateral vein using an insulin syringe 29-gauge under the camera to get the bolus. Four mice were injected with [⁶⁸Ga]Ga-JMV 7488 (6.58 ± 1.54 MBq, 4.3 ± 3.6 nmol, ~50 μL diluted in 150 μL of saline before administration), and four mice were injected with 0.5 μmol levocabastine 15 min prior to radiotracer injection (6.16 ± 1.21 MBq). PET acquisitions were done under anesthesia (isoflurane 3% induction and 1.5% maintenance in air) during 75 min. CT acquisitions were carried out at the end of the experiment. Animals were then sacrificed 90 min post-injection, and the organs were collected as described below.

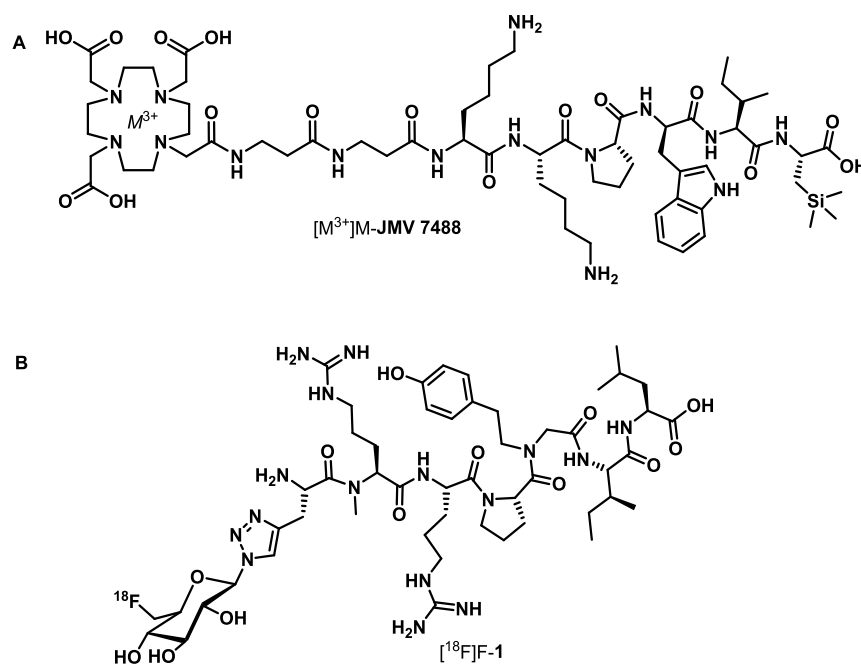


Figure 1. (A) Structure of $[M^{3+}]M$ -JMV 7488 developed in this study. M^{3+} stands for ^{68}Ga or ^{111}In in this study. Coordination bonds are not figured out. (B) Structure of the ^{18}F -labeled neurotensin peptoid $[^{18}F]F$ -1 previously developed in the literature to target the neurotensine receptor 2.

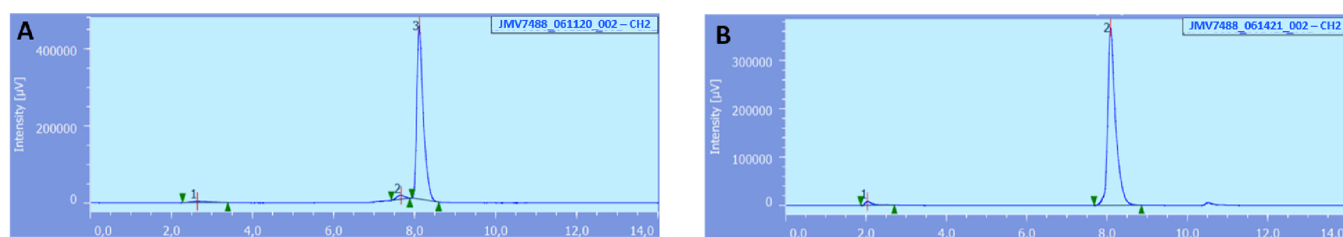


Figure 2. Radio-HPLC chromatogram of $[^{68}Ga]Ga$ -JMV 7488 (A) and $[^{111}In]In$ -JMV 7488 (B).

PET data were reconstructed using the OSEM 3D method. CT files were reconstructed using a Feldkamp algorithm after obtaining an isotropic voxel size of 0.170 mm.

Biodistribution Studies. Four mice were injected with $[^{68}Ga]Ga$ -JMV 7488 (6.58 ± 1.54 MBq, 4.3 ± 3.6 nmol, ~ 50 μ L diluted in 150 μ L of saline before administration), and four mice were injected with 0.5 μ mol levocabastine 15 min prior to radiotracer injection (6.16 ± 1.21 MBq). Solutions were injected into the tail vein. Animals were sacrificed 90 min post-injection by an intracardiac puncture. Blood, heart, lungs, liver, kidneys, spleen, stomach, intestines, muscle, bone, prostate, brain, and HT-29 tumor were collected. Organs were weighed and γ -counted (Wizard 2470, Perkin Elmer). Uptake levels were expressed as percentage of injected dose per gram of tissue (%ID/g).

Statistical Analyses. Statistical analyses were performed using Prism v6.01 software (GraphPad Software Inc., USA). Unpaired data were used. Quantitative values were expressed as means \pm SD. Statistical analysis with at least three groups were performed with the multiple t -tests corrected for multiple comparisons using the Holm Sidak method. Comparisons between two groups were performed using the bilateral nonparametric Mann–Whitney test. A P -value of <0.05 was considered significant.

Purity Statement. All compounds are $>95\%$ pure by HPLC and LCMS analysis. Representative traces are provided in the [Supporting Information](#).

RESULTS

Chemistry. The unnatural amino acid trimethylsilylalanine (TMSAla) carrying the fluorenylmethyloxycarbonyl (Fmoc) as the protecting group was synthesized following the synthetic pathway we already reported.²³ After that, the obtained Fmoc-TMSAla-OH was loaded on 2-chlorotriethyl chloride (2-CTC) resin,²⁴ which was previously treated with $SOCl_2$ to optimize the reactive sites. A loading of 0.8 mmol/g was detected with UV spectroscopy. On the contrary, the loading of Fmoc-TMSAla-OH resulted to be unproductive if untreated resin was used. The desired NT analogue JMV 7488 ([Figure 1](#)) was synthesized using the solid phase peptide synthesis (SPPS) approach with a yield of 31% (0.1 mmol scale). Even though the yield is moderately low, LC–MS analysis of the crude product after final cleavage showed a major peak with minimum amounts of by-products. This good HPLC profile allowed us to purify the desired product very easily. All data concerning the synthesis and characterization of JMV 7488 are detailed in the [Supporting Information](#).

^{68}Ga and ^{111}In Radiolabeling. Radiolabeling were achieved with moderate yields of 53.88 ± 8.34 (^{68}Ga , decay-

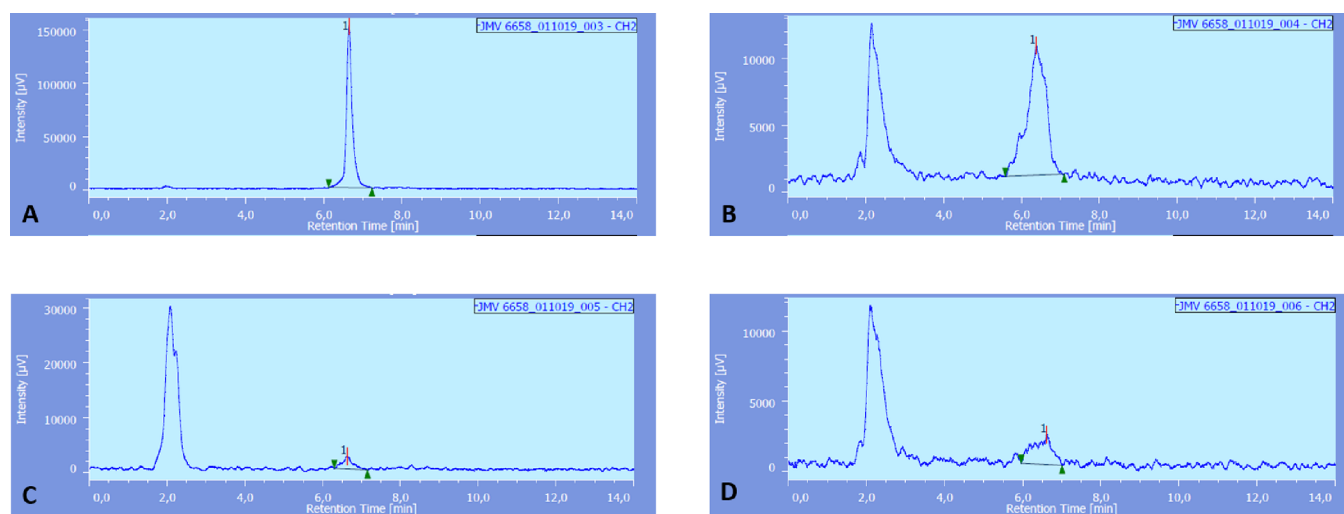


Figure 3. Radio-HPLC chromatograms of [^{68}Ga]Ga-JMV 7488 in human plasma 0 (A), 15 (B), 30 (C), and 45 min (D) after incubation.

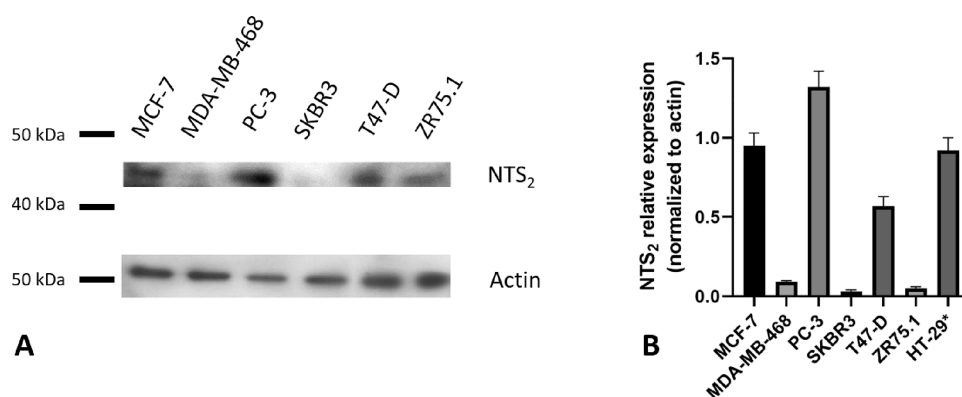


Figure 4. (A) Representative western blot of the NTS₂ receptor on cell lysate from MCF-7, MDA-MB-468, PC-3, SKBR3, T47-D, and ZR75.1 cells. (B) Semi-quantification of the NTS₂ signal from the western blots. (*) Data regarding NTS₂ expression on HT-29 cells are issued from our previous work.²⁴ Adapted with permission from Fanelli et al. *Bioconjugate Chemistry* 2020, 31, 10, 2339–2349. Copyright 2020 American Chemical Society.

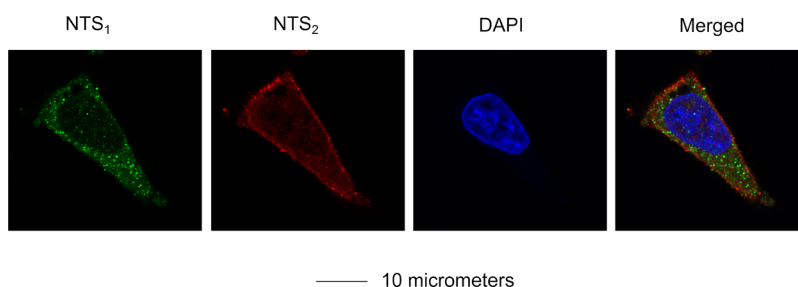


Figure 5. Confocal imaging of neurotensin receptors NTS₁ and NTS₂ on MCF-7 cells using indirect immunofluorescence. Images were obtained at 63 \times magnification. (NTS₁) intracytoplasmic granular expression of the NTS₁ receptor in MCF-7 cells. (NTS₂) Membrane expression of the NTS₂ receptor in MCF-7 cells. (DAPI) Nucleus staining of alive MCF-7 cells using DAPI. (merged) Combination of NTS₁, NTS₂, and DAPI signal in MCF-7 cells.

corrected) and $63.75 \pm 12.42\%$ (^{111}In , decay corrected), high volumetric activities of 108.86 ± 15.24 (^{68}Ga) and 39.20 MBq/mL (^{111}In), and apparent molar activities of 8.77 ± 1.23 (^{68}Ga) and 2.95 GBq/ μmol (^{111}In). Radiochemical purities were always >95%. No degradation of the radiolabeled product was observed in the vehicle over 8 days (on radio-HPLC analysis using the ^{111}In radiolabeled compound, Figure 2).

Human Blood Plasma Stability. Stability in human blood plasma was performed using [^{68}Ga]Ga-JMV 7488. At 15 min,

23.5% of the peptide remained intact and this value decreased up to 5% after 30 min (Figure 3).

In Vitro Studies. NTS₂ expression has already been reported on HT-29 cells in previous studies.²⁴ To get further insight in the potential of NTS₂ in oncology, we performed western blot experiments on five breast cancer cell lines (MCF-7, MDA-MB-468, SKBR3, T47-D, and ZR75.1), on the prostate cancer cell line PC-3, and on the colorectal adenocarcinoma cell line HT-29. Semi-quantification of the

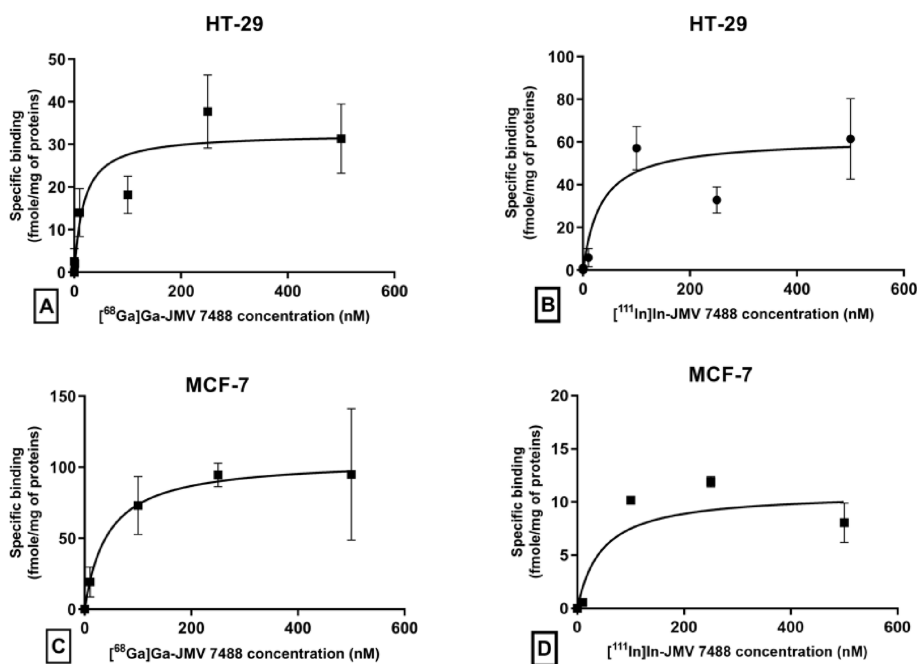


Figure 6. Saturation binding curves of $[^{68}\text{Ga}]\text{Ga-JMV 7488}$ on HT-29 cells (A) and MCF-7 cells (C) and $[^{111}\text{In}]\text{In-JMV 7488}$ on HT-29 cells (B) and MCF-7 cells (D) toward NTS_2 . Blocking concentration of levocabastine = $1 \mu\text{M}$.

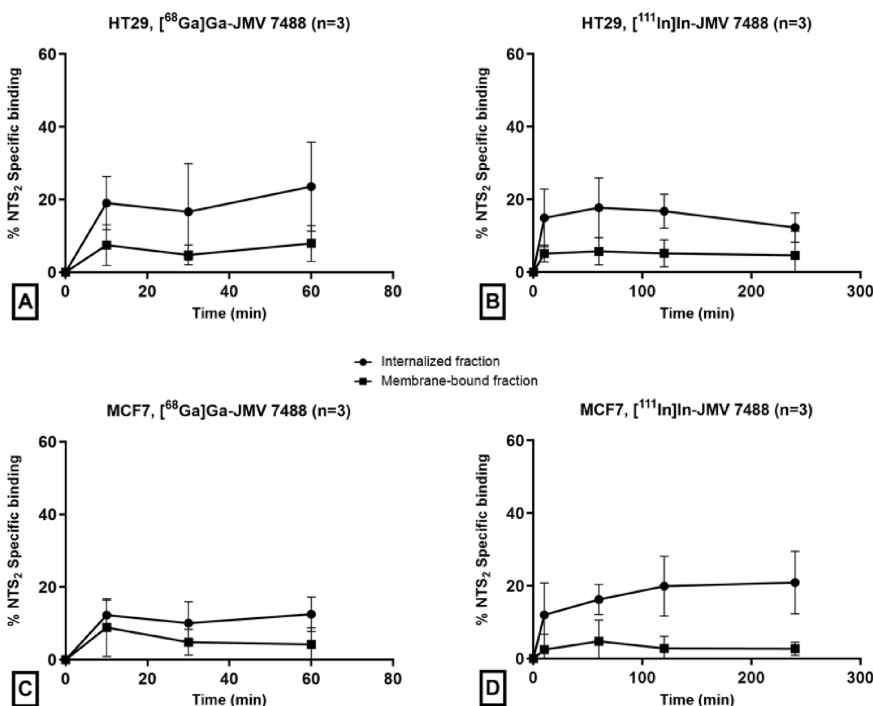


Figure 7. NTS_2 mediated internalizations of $[^{68}\text{Ga}]\text{Ga-JMV 7488}$ on HT-29 (A) and MCF-7 (C) cells and $[^{111}\text{In}]\text{In-JMV 7488}$ on HT-29 (B) and MCF-7 (D) cells.

western blot showed that among the breast cancer cell line, the MCF-7 cells do express the highest level of NTS_2 . HT-29 cells express the NTS_2 receptor. Lastly, PC-3 cells express the highest amount of NTS_2 among explored cancer cell lines (Figure 4).

Next, we performed confocal imaging on MCF-7 cells to assess the intracellular pattern of expression of NTS_2 . As a validation step, we confirmed that MCF-7 cells express NTS_1 and that staining was intracytoplasmic and granular. Interest-

ingly, MCF-7 cells express NTS_2 and the staining was mostly membranous as depicted in Figure 5. Negative controls did not exhibit staining.

Lipophilicity. $\text{LogD}_{7.4}$ values were -3.11 ± 0.24 for $[^{68}\text{Ga}]\text{Ga-JMV 7488}$ and -2.67 ± 0.18 for $[^{111}\text{In}]\text{In-JMV 7488}$. Values were statistically significant ($p < 0.0001$).

Affinity Studies. Saturation binding studies performed on HT-29 and MCF-7 cells revealed that both $[^{68}\text{Ga}]\text{Ga-JMV 7488}$ and $[^{111}\text{In}]\text{In-JMV 7488}$ displayed a good affinity at

NTS₂ (Figure 6) and no specific binding at NTS₁ up to 0.5 μM. K_D values obtained were 37.83 ± 17.20 nM on HT-29 cells and 36.37 ± 9.89 nM on MCF-7 cells for [⁶⁸Ga]Ga-JMV 7488; 36.39 ± 4.02 nM on HT-29 cells and 45.94 ± 0.60 nM on MCF-7 cells for [¹¹¹In]In-JMV 7488. Bmax values at the NTS₂ receptor were 30.30 ± 18.46 fmol/mg of proteins on HT-29 cells and 50.15 ± 40.36 fmol/mg of proteins on MCF-7 cells (mean value obtained with [⁶⁸Ga]Ga-JMV 7488 and [¹¹¹In]In-JMV 7488).

Cell-Associated Radioactivity and Internalization Studies. After 1 h incubation at 37 °C, NTS₂-mediated uptakes (internalized and membranous) were 23.98 ± 4.79% of total binding for [⁶⁸Ga]Ga-JMV 7488 on HT-29 cells and 12.88 ± 8.23% on MCF-7, and for [¹¹¹In]In-JMV 7488, 19.72 ± 9.72 on HT-29 and 25.18 ± 11.73% on MCF-7 cells.

Kinetic studies (Figure 7) indicated that the internalized fraction rapidly reached its maximum 10 min after incubation and that this value remained stable over the experiment timeline (60 min for ⁶⁸Ga, 240 min for ¹¹¹In). The membrane-bound fraction was always below 8% of total binding.

Specific internalization represented 13.85 ± 9.75% of applied dose (AD) on HT-29 cells and 13.79 ± 4.78% AD on MCF-7 cells at 60 min.

Efflux Studies. [¹¹¹In]In-JMV 7488 demonstrated an intense efflux as early as 5 min (68.29 ± 19.30% on HT-29 cells and 54.34 ± 16.54% on MCF-7 cells). This value increased up to 78.06 ± 16.64% on HT-29 and 74.97 ± 9.56% on MCF-7 cells at 60 min and remained stable until 120 min (Figure 8). In prevision of *in vivo* experiments, efflux studies

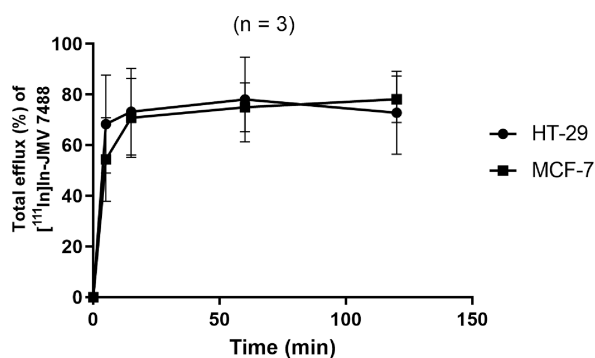


Figure 8. Total efflux of [¹¹¹In]In-JMV 7488 on HT-29 and MCF-7 cells.

were also performed with the gallium-68 radiolabeled compound on HT-29 cells. A similar behavior was seen with a high efflux rate of 45.28 ± 6.85% at 5 min, reaching 66.06 ± 8.05% at 45 min (data not shown).

Calcium Imaging Assay. Concentration-dependent Ca²⁺ mobilization was investigated with JMV 7488 and the NTS₂ agonist levocabastine as the positive control. EC₅₀ values were 431 and 118 nM for JMV 7488 and levocabastine, respectively (Figure 9). JMV 7488 was able to promote the levocabastine-induced maximum dose–response (90.88 ± 11.49%), meaning that JMV 7488 is a full agonist at the NTS₂.

***In Vivo* Studies. Biodistribution Study.** Four mice were injected with 6.58 ± 1.54 MBq of [⁶⁸Ga]Ga-JMV 7488 and sacrificed 90 min after injection. Four other mice were co-injected with levocabastine (LV) along with 6.16 ± 1.21 MBq of [⁶⁸Ga]Ga-JMV 7488 to demonstrate the specificity of the binding. [⁶⁸Ga]Ga-JMV 7488 exhibited nonspecific uptake in

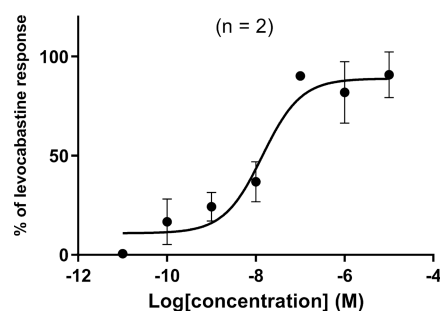


Figure 9. Intracellular mobilization of Ca²⁺ induced by JMV 7488. Results are normalized from stimulation induced by levocabastine.

kidneys (30.1 ± 18.4% ID/g) and prostate (2.25 ± 0.65% ID/g). Interestingly, HT-29 tumor and lungs showed specific uptake of [⁶⁸Ga]Ga-JMV 7488. The tumor/muscle ratio was significantly decreased when levocabastine was injected (5.5 ± 1.3 vs 1.7 ± 1.9 %ID/g, *p* = 0.02). Other organs did not show significant uptake (Figure 10 and Supporting Information Table S1).

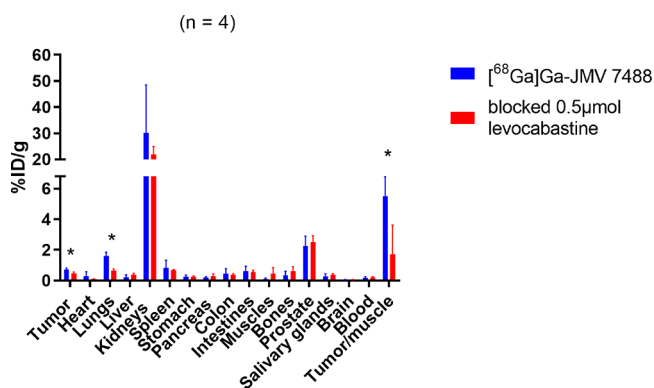


Figure 10. Biodistribution of [⁶⁸Ga]Ga-JMV 7488 injected in HT-29 xenografted mice. * indicates statistical differences (*p* < 0.05).

PET/CT Imaging. Whole-body μPET/CT imaging was performed on nude mice bearing HT-29 xenograft injected with [⁶⁸Ga]Ga-JMV 7488 without and with excess of levocabastine to assess NTS₂-specificity *in vivo*. Elimination of the radiotracer was urinary-exclusive. Unfortunately, no evident uptake on HT-29 was depicted on the PET/CT images at any time point (Figure 11).

DISCUSSION

Targeting neurotensin and its receptors holds promises in nuclear oncology.²⁵ The NTS₁ receptor has been shown to be over-expressed in several poor prognosis cancers such as pancreatic adenocarcinoma, triple negative breast cancer and glioblastoma among others.^{26–28} However, little is known regarding the expression of the NTS₂ subtype in tumors and very few targeting biomolecules have been designed to target the NTS₂ subtype.¹⁸ Thus, the aim of this work was to synthesize and characterize a new NTS₂-targeted radiolabeled peptide. As preliminary steps, NTS₂ expression was assessed in a panel of breast cancer cell lines and on a prostate cancer cell line using western blot. Results showed that expression of NTS₂ is variable across breast cancer cell lines, its over-expression being noticed in less aggressive phenotypes represented by MCF-7 and T47D cells (luminal A phenotype).

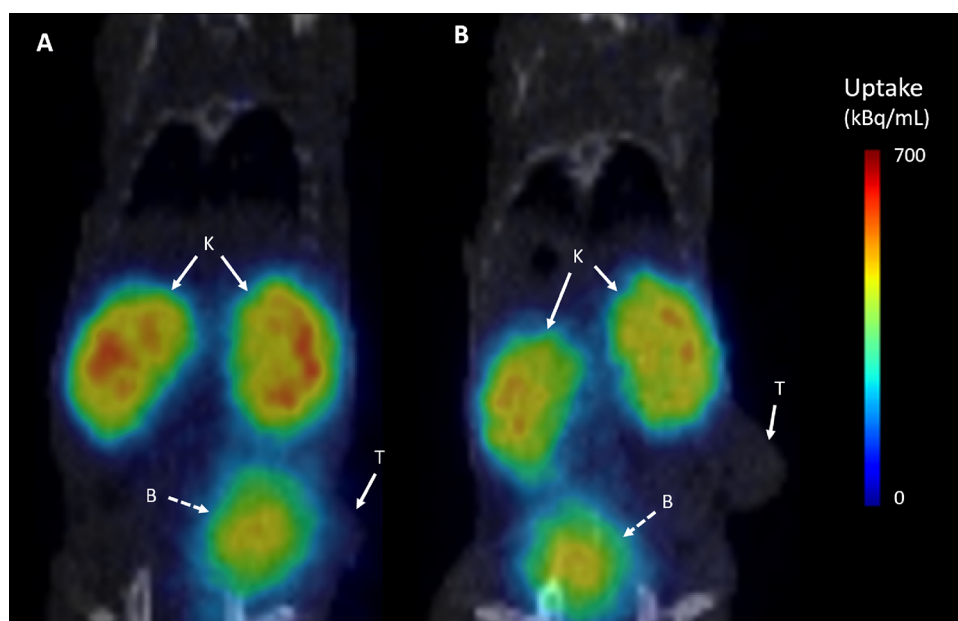


Figure 11. μ PET/CT imaging of [^{68}Ga]Ga-JMV 7488 in HT-29 tumor-bearing nude mice. (A) Fused coronal PET/CT image at 75 min post-injection of a nude mice injected with 6 MBq of [^{68}Ga]Ga-JMV 7488 alone. (B) Fused coronal PET/CT image at 90 min post-injection of a nude mice co-injected with 6 MBq of [^{68}Ga]Ga-JMV 7488 and excess of levocabastine (0.5 μmole). K stands for kidneys, B is the bladder, and T is the HT-29 xenografted tumor.

Confocal imaging of NTS₁/NTS₂ expression was then assessed on the MCF-7 cell line, and the staining was membranous (Figure 4), which confirmed the rapid recycling properties of NTS₂ to the cell membrane upon stimulation.²⁹ In prostate cancer, the PC-3 cell line express an even higher amount of NTS₂. At last, we previously demonstrated that the colon cancer cell line HT-29 does express the NTS₂ subtype suggesting the potential of NTS₂ for imaging cancer types needing novel imaging strategies.²⁴ In this study, we were interested in developing a specific NTS₂-radiolabeled peptide (i.e., JMV 7488). HT-29 and MCF-7 cells were chose as models for preclinical characterization because (1) HT-29 is the standard cell line to characterize NTS₁-analogues, therefore allowing comparison, and (2) the MCF-7 cells, representing the luminal A breast cancer phenotype, need novel imaging agents to overpass intrinsic limitations of [^{18}F]-FDG.

Structurally, JMV 7488 is composed by a chelating agent (DOTA), two β -alanine (βAla) residues, and a modified NT sequence. While several chelating agents have been investigated,³⁰ DOTA is largely used for the development of radiopharmaceuticals.³¹ The role of the linker between the chelating agents and the sequences responsible for the binding was widely discussed.^{32,33} In JMV 7488, we inserted two βAla residues, with the aim to distance DOTA and the NT sequence. Regarding the latter, we and others identified the residue at the position 11 as being crucial for selectivity.^{14,34–39} Considering the double interest of silicon-containing amino acids that confer both resistance to enzyme degradation and lipophilicity,⁴⁰ we also demonstrated that the replacement of Leu at position 13 by a TMSAla enhanced the binding affinity of NT ligands for both NTS₁ and NTS₂ by improving the interactions with the hydrophobic ligand binding pocket of both receptors.⁴¹ More recently, we developed a series of modified NT analogues, in which some of them showed a NTS₁-mediated hypothermic effect.²² Among all the analogues, the hexapeptide JMV 5504 (H-Lys-Lys-Pro-(D)Trp-

Ile-TMSAla-OH) showed the best selectivity toward hNTS₂, with Ki values of 8.5 and 3600 nM toward hNTS₂ and hNTS₁, respectively (selectivity index hNTS₁/hNTS₂ = 423). Considering the poor NTS₁ binding affinity, no hypothermic effect for JMV 5504 was observed. In the light of our findings, the modified NT sequence of JMV 5504 was selected for the development of the first radiolabeled peptide targeting NTS₂ in peripheral tumors. The SPPS approach used for the synthesis of the desired product resulted to be really efficient, fast, and highly reproducible. After Fmoc-TMSAla-OH loading on 2-CTC resin, Fmoc-deprotections and couplings were carried out as we reported in detail in the Supporting Information. In this context, it has to be noted that the Kaiser test showed an uncomplete coupling reaction when Fmoc-Pro-OH, Fmoc-Lys-OH (for position 10), and DOTA(OtBu)₃-OH were used. For these reasons, the three above-mentioned coupling reactions were performed in duplicate. The final cleavage resulted to be very efficient after 7 h in vigorous shaking; meanwhile, shorter times have proved to be insufficient for a complete deprotection. In particular, by-products bearing -OtBu protecting groups were observed. After the cleavage, the desired NT analogue JMV 7488 was purified and characterized.

We first looked at the physicochemical parameters of [^{68}Ga]Ga-JMV 7488 and [^{111}In]In-JMV 7488 using the octanol/PBS partition coefficient method. We found that both radiotracers exhibit a marked hydrophilicity at pH 7.4, consistent with previous neurotensin analogues. Blood plasma stability of [^{68}Ga]Ga-JMV 7488 was tested on human plasma, and rapid cleavage was demonstrated (Figure 3). Strategies to improve the stability need to be carried out. They might include the use of enzyme inhibitors,⁴² structural modification of the C-terminal hexapeptide binding sequence,⁴³ modification of the linker/chelate,³⁰ etc.

Radiolabeled JMV 7488 Were Then Investigated *In Vitro* on HT-29 Cells and MCF-7 Cells. Results from

saturation binding experiments showed that both radiolabeled bioconjugates exhibit a quite lower NTS₂ selectivity compared to the unconjugated counterpart JMV 5504.²² Addition of the M³⁺-DOTA-(βAla)₂ moiety to the binding sequence results in the moderate loss of NTS₂ affinity (8.5 ± 2 nM [Ki] for JMV 5504 vs 38 ± 17 nM on HT-29 cells and 36 ± 10 nM on MCF-7 cells for [⁶⁸Ga]Ga-JMV 7488; 36 ± 4 nM on HT-29 cells and 46 ± 1 nM on MCF-7 cells for [¹¹¹In]In-JMV 7488). Interestingly, no NTS₁-binding was seen for both radiotracers indicating good selectivity (concentrations higher than 0.5 μM were not tested). The total number of NTS₂ receptor on HT-29 cells obtained by saturation binding experiment was consistent with previously published data.⁴⁴ We acknowledge that, compared to the ¹⁸F-labeled NTS₂ glycopeptide ¹⁸F-1,¹⁸ the NTS₂ affinities of our compounds were somewhat lower, but our radiolabeled peptides pave the way for the first time to NTS₂-directed imaging and treatment thanks to the DOTA macrocycle suitable for radiolabeling with radiometals for PET and/or TRT. This is not achievable with fluorine chemistry. Two other lessons can be deduced from these experiments. First, NTS₂ binding is not sensitive to hydrophilicity as [¹¹¹In]In-JMV 7488 was significantly less hydrophilic than [⁶⁸Ga]Ga-JMV 7488 without impact on NTS₂-binding. Second, HT-29 and MCF-7 cells provide same biological responses, indicating that breast cancer represents also an interesting model to study NTS₂-directed radiotracers. We encourage further studies in this setting.

Next, we investigated the intracellular behavior of [⁶⁸Ga]Ga-JMV 7488 in HT-29 cells and [¹¹¹In]In-JMV 7488 on MCF-7 cells. The high internalization rate of 20.53 ± 6.50% for [⁶⁸Ga]Ga-JMV 7488 is in line with its NTS₂ affinity. This value might appear low compared to the internalization capacity of NTS₁, which is about 70%, but one should keep in mind that the internalization process is the combination of several mechanisms including the β-arrestin coupling of the receptor and the recycling properties of the receptor. In the specific case of the NTS₂ receptor, β-arrestin coupling reached 85% of the specific binding similarly to NTS₁ but NTS₂ is rapidly recycled to the membrane contrarily to NTS₁, which is intracellularly retained.²⁹ Collectively, these data seem to indicate that our compounds promote high NTS₂-internalization, meaning that they behave like agonists regarding the NTS₂. The low membrane-bound fractions illustrate also well the supposed agonist behavior of [⁶⁸Ga]Ga-JMV 7488 and [¹¹¹In]In-JMV 7488 since agonists are internalized while antagonists usually do not. However, as the possibility for antagonists to undergo internalization has been highlighted,⁴⁵ we went deeper in its biological characterization by performing intracellular calcium mobilization experiments (Figure 9). As preliminary steps, we looked at the intracellular calcium mobilization of levocabastine as this point is still matter of debate⁴⁶ and was not explored in HT-29 cells. For the first time, we demonstrate that levocabastine is able to induce mobilization of intracellular calcium. Moreover, efficacy of JMV 7488 reaches the maximum intracellular calcium mobilization promoted by levocabastine, indicating that it is a full agonist at the NTS₂. The lower EC₅₀ for JMV 7488 (411 vs 118 nM) reflects the lower NTS₂ affinity compared to levocabastine (this experiment was performed using the nonlabeled bioconjugate JMV 7488).

Efflux experiments were finally conducted, given the internalization of [⁶⁸Ga]Ga-JMV 7488 and [¹¹¹In]In-JMV 7488. Our results showed a high efflux, for both radiotracers

(Figure 8). Overall, this is the first detailed *in vitro* characterization of a NTS₂-directed radiolabeled peptide for peripheral tumor imaging.

Because HT-29 tumors grow fast and do not require hormone supplementation and because of the possibility of quantification using PET, we choose to investigate [⁶⁸Ga]Ga-JMV 7488 *in vivo* in nude mice bearing HT-29 xenograft with the ⁶⁸Ga-labeled compound. Specificity of the binding was confirmed by displacement of 44% of [⁶⁸Ga]Ga-JMV 7488 signal by the NTS₂-preferring agonist levocabastine (full displacement was not reached). In biodistribution experiments performed 90 min after injection, uptake of [⁶⁸Ga]Ga-JMV 7488 in HT-29 tumor was still 0.72 ± 0.10%ID/g. Co-injection of levocabastine significantly decreased this value to 0.45 ± 0.10%ID/g, *p* = 0.03. In comparison with the ¹⁸F-1 agent developed by Prante's lab,¹⁶ our compound provided a higher uptake in tumor at a later time point (90 min vs 60 min), which is a significant improvement. As expected, given the hydrophilicity of the radiotracer, [⁶⁸Ga]Ga-JMV 7488 was eliminated by the kidneys (Figure 11). Unfortunately, the uptake on HT-29 tumor was too low to build an image on the PET/CT presumably due to its low stability. The development of NTS₂-specific radiotracers allows a better knowledge of the distribution of NTS₂, and our study went deeper in this characterization compared to the literature. Our results provide for the first time evidence of an uptake [⁶⁸Ga]Ga-JMV 7488 in the mice prostate (translation to humans must be done carefully in the same way as the prostate specific membrane antigen (PSMA), which is expressed in mouse kidneys but absent in human kidneys). Although this nonspecific mechanism remains to be explored in details, this finding represents a caveat for the use of orthotopic models of primary prostate cancer to investigate NTS₂-targeting agents. Nevertheless, considering that in metastatic prostate cancer, tumor cells are disseminated outside the prostate bed, and that NTS₂ is expressed in PSMA-negative metastases (*i.e.*, PC-3 cells¹⁸ and our data in this work), NTS₂-directed theranostic is appealing in case of [¹⁷⁷Lu]Lu-PSMA remains ineffective.⁴⁷ Moreover, NTS₂ is absent from salivary glands, which physiologically express the PSMA, therefore explaining xerostomia (partially) responsible for treatment discontinuation when using radiolabeled PSMA-inhibitors.⁴⁸ Moreover, the lungs showed moderate uptake of [⁶⁸Ga]Ga-JMV 7488 in line with the known expression of NTS₂.²¹ Other organs do not retain significant uptake and therefore NTS₂ expression.

CONCLUSIONS

Collectively, targeting the NTS₂ subtype appears highly promising in phenotypes lacking imaging/therapeutic procedures such as low-FDG avid breast cancer cells, PSMA-negative prostate cancer cells, and colorectal cancer cells. Subsequent studies will further explore the expression profile of NTS₂ in human tumors and healthy tissues.

[⁶⁸Ga]Ga-JMV 7488 and [¹¹¹In]In-JMV 7488 showed good affinity and high internalization rate at the NTS₂. *In vivo*, [⁶⁸Ga]Ga-JMV 7488 showed moderate uptake on HT-29 tumors. Radiolabeled JMV 7488 behaves as a promising tool to assess NTS₂ expression in periphery. Further improvements in tumoral uptake and stability remain needed.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c07814>.

Synthesis, analytical characterization of the desired NT analogue JMV 7488, radiolabeling procedures, determination of the octanol/water partition coefficient, cell culture, confocal imaging, *ex vivo* stability methods, and *ex vivo* biodistribution (PDF)

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Author Contributions

*F.C. and C.M. contributed equally to directing this study. S.P. and E.R. synthesized and purified the JMV 7488 compound. S.B., D.V., and C.M. performed radiolabeling and *in vitro* experiments and analyzed data. I.A.A. and E.J. performed *in vivo* experiments. F.L. analyzed microPET data. E.H. critically reviewed the manuscript. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

⁶⁸Ga, gallium 68; ¹¹¹In, indium-111; FBS, fetal bovine serum; GPCR, G protein-coupled receptors; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; NT, neurotensin; NTS₁, NT receptor 1; NTS₂, NT receptor 2; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TMSAla, (L)-(trimethylsilyl)alanine.

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