

DOTA-PESIN, a DOTA-conjugated bombesin derivative designed for the imaging and targeted radionuclide treatment of bombesin receptor-positive tumours

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Abstract

Purpose We aimed at designing and developing a novel bombesin analogue, DOTA-PEG₄-BN(7–14) (DOTA-PESIN), with the goal of labelling it with ^{67/68}Ga and ¹⁷⁷Lu for diagnosis and radionuclide therapy of prostate and other human cancers overexpressing bombesin receptors.

Methods The 8-amino acid peptide bombesin (7–14) was coupled to the macrocyclic chelator DOTA via the spacer 15-amino-4,7,10,13-tetraoxapentadecanoic acid (PEG₄). The conjugate was complexed with Ga(III) and Lu(III) salts. The GRP receptor affinity and the bombesin receptor subtype profile were determined in human tumour specimens expressing the three bombesin receptor subtypes. Internalisation and efflux studies were performed with the human GRP receptor cell line PC-3. Xenografted nude mice were used for biodistribution.

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Results [⁶⁷Ga/¹⁷⁷Lu]-DOTA-PESIN showed good affinity to GRP and neuromedin B receptors but no affinity to BB3. [⁶⁷Ga/¹⁷⁷Lu]-DOTA-PESIN internalised rapidly into PC-3 cells whereas the efflux from PC-3 cells was relatively slow. In vivo experiments showed a high and specific tumour uptake and good retention of [⁶⁷Ga/¹⁷⁷Lu]-DOTA-PESIN. [⁶⁷Ga/¹⁷⁷Lu]-DOTA-PESIN highly accumulated in GRP receptor-expressing mouse pancreas. The uptake specificity was demonstrated by blocking tumour uptake and pancreas uptake. Fast clearance was found from blood and all non-target organs except the kidneys. High tumour-to-normal tissue ratios were achieved, which increased with time. PET imaging with [⁶⁸Ga]-DOTA-PESIN was successful in visualising the tumour at 1 h post injection. Planar scintigraphic imaging showed that the ¹⁷⁷Lu-labelled peptide remained in the tumour even 3 days post injection. **Conclusion** The newly designed ligands have high potential with regard to PET and SPECT imaging with ^{68/67}Ga and targeted radionuclide therapy with ¹⁷⁷Lu.

Keywords Gallium-68 · Lutetium-177 ·
Bombesin receptors · Targeted radiotherapy ·
Prostate cancer

Introduction

Radionuclides coupled to receptor-specific peptides are currently under investigation in clinical trials involving different tumours [1]. They specifically localise receptors overexpressed on the plasma membrane and then internalise into cells [2–4]. The prototypes of these peptides are analogues of somatostatin [5].

In designing radiometal-based radiopeptides for cancer diagnosis and treatment, important factors to consider are

half-life, mode of decay, cost and availability of the radionuclide. In diagnostic imaging [6], ^{99m}Tc , ^{123}I , ^{67}Ga and ^{111}In are used for SPECT and ^{18}F , ^{11}C , ^{64}Cu and ^{68}Ga for positron emission tomography (PET). ^{177}Lu -labelled peptides have become attractive in targeted radiotherapy of small tumours or metastases owing to their excellent radiophysical properties and commercial availability of the radionuclide [5, 7, 8]. ^{68}Ga , on the other hand, is a promising metallic positron emitter ($t_{1/2}=68$ min, 89%, $E_{\beta^+ \text{ max}}=1.90$ MeV) for regular use in PET imaging because of its production from a $^{68}\text{Ga}/^{68}\text{Ge}$ generator, which allows independence from an on-site cyclotron and offers almost unlimited availability if two or three of these generators are used concomitantly as elution is possible every 3 h [9, 10]. To develop a ligand for the labelling with these two radionuclides would therefore seem promising.

In the field of radiolabelled peptides, bombesin (BN) analogues have become a promising class of ligands as gastrin-releasing peptide (GRP) receptors, a subtype of BN receptors, were found to be overexpressed on primary prostatic invasive carcinoma (and their invaded lymph nodes), breast tumours and gastrointestinal stromal tumours [11]. Up to now, many types of radiolabelled BN analogues have been designed to target GRP receptor-expressing tumours [12–24]. In particular, Van de Wiele et al. reported on a first study of prostate ($n=4$) and breast cancer ($n=6$) patients [13, 25]. Their results showed that ^{99m}Tc -RP527 visualised four of six breast and one of four prostate carcinomas. This radiopeptide showed inferior properties to many other radiopeptides in the PC-3 tumour-bearing nude mouse model [26]. As ^{99m}Tc -Demobesin 1 [^{99m}Tc -N4 $^{0-1}$, bzlg 0 , (D)Phe 6 , Leu-NHET 13 , *des*-Met 14]BN(6–14)) has shown higher affinity to the GRP receptor and a higher tumour accumulation in PC-3 tumour-bearing mice [27] than ^{99m}Tc -RP527, it may be a superior candidate for studying in patients.

We have recently developed DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)- and DTPA-modified (pan)BN radioligands having high affinity to all three human BN receptors [i.e. neuromedin B receptor (NMB-R), GRP-R and BN receptor subtype-3 (BB3)] [17]. Preliminary results in clinical studies of two of these radiopeptides have shown occasional uptake in the pancreas and relatively fast washout from lesions when labelled with ^{111}In . The fast washout may be due to a low metabolic stability of these radiopeptides. Therefore, we set up a programme to synthesise BN-based radiopeptides with improved metabolic stability, an optimised tumour-to-kidney ratio and lack of affinity to BB3. From a series of peptides evaluated, DOTA-PEG $_4$ -BN(7–14) (DOTA-PESIN) looked particularly promising in our preclinical studies.

Here we present data on the synthesis and labelling of this peptide with the positron emitter ^{68}Ga , the γ - and

Auger electron emitter ^{67}Ga and the $\beta(\gamma)$ -emitter ^{177}Lu . In addition, we studied the receptor subtype affinity profile and the in vivo biodistribution of radiolabelled DOTA-PESIN with regard to BN receptor subtypes. PET imaging and scintigraphy were also performed.

Materials and methods

All chemicals were obtained from commercial sources and used without further purification. Rink amide 4-methylbenzhydrylalanine (MBHA) resin and all Fmoc-protected amino acids are commercially available from NovaBiochem (Laufelfingen, Switzerland). [^{67}Ga]Cl $_3$ was purchased from Mallinckrodt Medical (Petten, the Netherlands) and [^{177}Lu]Cl $_3$ from IDB (Petten, the Netherlands). Fmoc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (Fmoc-PEG $_4$ -OH) was obtained from Quanta BioDesign (Powell, Ohio, USA) and 1,4,7,10-tetraazacyclododecane-1,4,7-*tris*(acetic acid-*t*-butyl ester)-10-acetic acid [DOTA-*tris*(t -Bu ester)] from Macrocyclics (Dallas, Texas, USA). Electrospray ionisation mass spectroscopy was carried out with a Finnigan SSQ 7,000 spectrometer (Bremen, Germany) and MALDI-MS measurement on a Voyager sSTR equipped with an Nd:YAG laser (Applied Biosystems, Framingham, USA). Ten milligrams of 3,5-dimethoxy-4-hydroxycinnamic acid were dissolved in 0.05% TFA of acetonitrile/water (1:1) and used as matrix. Analytical high-performance liquid chromatography (HPLC) was performed on a Hewlett Packard 1050 HPLC system with a multiwavelength detector and a flow-through Berthold LB 506 Cl γ -detector (Regensdorf, Switzerland) using a Macherey-Nagel Nucleosil 120 C $_{18}$ column (Oensingen, Switzerland) (eluent: A=0.1% TFA in water and B=acetonitrile; gradient: 0–20 min, 80%–50% A; 21–24 min, 100% B; 24–25 min, 80% A). Preparative HPLC was performed on a Metrohm HPLC system LC-CaDI 22–14 (Herisau, Switzerland) with a Macherey-Nagel VP 250/21 Nucleosil 100–5 C $_{18}$ column (eluent: A=0.1% TFA in water and B=acetonitrile; gradient: 0–10 min, 70%–52% A; 11–14 min, 100% B; 14–15 min, 70% A). Quantitative γ -counting was performed on a COBRA 5003 γ -system well counter from Packard Instruments (Meriden, CT, USA). Solid phase peptide synthesis was performed on a semiautomatic peptide synthesiser commercially available from Rink CombiChem Technologies (Bubendorf, Switzerland). The cell culture medium was Dulbecco's minimal essential medium (DMEM) with 10% or 1% foetal calf serum (FCS) from BioConcept (Allschwil, Switzerland). PET imaging was performed on a Siemens ECAT EXACT HR $^+$ scanner (Siemens/CTI, Knoxville, TN, USA), and scintigraphic imaging was carried out on a PRISM 2000 XP camera (Philips, the Netherlands). ^{68}Ga was obtained from a

$^{68}\text{Ge}/^{68}\text{Ga}$ generator which consists of a column containing a self-made phenolic ion exchanger loaded with ^{68}Ge and coupled in series with a small anion exchanger column (AG 1 \times 8 Cl-1, mesh 200–400; Bio-Rad, Munich, Germany) to concentrate ^{68}Ga during elution [28].

Synthesis

DOTA-PESIN was synthesised on solid support as described previously [17]. In brief, the peptide was assembled on a Rink amide MBHA resin according to classical Fmoc chemistry on a semi-automatic peptide synthesiser. Trityl and *tert*-butoxycarbonyl were used as side chain-protecting groups of His and Trp, respectively. Fmoc-PEG₄-OH (four equivalents) and DOTA-*tris*(tBu) ester (three equivalents) were consecutively coupled to the peptide with HATU [1.2 equivalents based on Fmoc-PEG₄-OH or DOTA-*tris*(tBu) ester] as activating agent. Cleavage from the resin and deprotection as well as product purification and identification were performed according to a previously described protocol [17]. Calculated MW: 1573.81; MS-ES(-): 1610.3 [M+K-H]⁻, 804.8 [M+K-2H]²⁻; MS-ES(+): 806.8 [M+K+2H]²⁺; MALDI, m/z (%): 1573.7 (100, [M+H]⁺), 1594.7 (5, [M+Na]⁺); HPLC t_R: 12.5 min on analytical HPLC (purity: >98%).

[$^{67}\text{Ga}^{\text{III}}/\text{Lu}^{\text{III}}$]-DOTA-PESIN A mixture of DOTA-PESIN (0.5 μmol) in 500 μl 0.5 mol/l ammonium acetate buffer (pH 5) was incubated with 1.5 μmol Ga(NO₃)₃·9H₂O or Lu(NO₃)₃·5H₂O (in 0.04 mol/l HCl) at 95°C for 25 min, and purified over a SepPak C₁₈ cartridge (Waters Corp., Milford, MA) preconditioned with 10 ml ethanol and 10 ml water. The cartridge was eluted with 10 ml water followed by 3 ml methanol, resulting in the metal^{III}-DOTA-peptide after evaporation of methanol. The final product was analysed by analytical HPLC and MALDI. [$^{67}\text{Ga}^{\text{III}}$]-DOTA-PESIN (calculated MW): 1640.51; MALDI, m/z (%): 1641.7 (100, [M+H]⁺), 1663.6 (20, [M+Na]⁺); HPLC t_R: 13.8 min. Purity: >99%. [$^{\text{III}}\text{Lu}$]-DOTA-PESIN (calculated MW): 1745.76; MALDI, m/z (%): 1745.8 (100, [M+H]⁺), 1767.7 (10, [M+Na]⁺); HPLC t_R: 14.1 min. Purity: >99%.

Preparation of radiotracer for in vitro and in vivo studies

[^{177}Lu]-DOTA-PESIN was prepared by dissolving 10 μg of the peptide (6.35 nmol) in ammonium acetate buffer (300 μl , 0.5 mol/l, pH 5.5); after addition of $^{177}\text{LuCl}_3$ (2 mCi), the solution was incubated for 15 min at 95°C. A 1.5 molar excess of Lu(NO₃)₃·5H₂O was added and incubated again for 15 min. Subsequently, [^{177}Lu]-DOTA-PESIN was purified utilising a SepPak C₁₈ cartridge

preconditioned with 10 ml methanol and 10 ml water; the cartridge was eluted with 3 ml water, followed by 2 ml ethanol to yield pure [^{177}Lu]-DOTA-PESIN. For biodistribution studies, the labelling was performed accordingly without the addition of cold Lu(NO₃)₃·5H₂O. Oxidation of C-terminal MetNH₂ was suppressed by the addition of methionine. The solution for injection was prepared by dilution with 0.9% NaCl (0.1% BSA) to provide the radioligand solution. [^{67}Ga]-DOTA-PESIN was prepared in the same way. All radiolabelled peptides were analysed with analytical HPLC and showed a radiochemical purity of >97%.

Preparation of [^{68}Ga]-DOTA-PESIN for PET imaging

The generator eluate containing ~0.5 GBq ^{68}Ga in 0.2 ml/0.5 ml/l HCl was evaporated to dryness and redissolved in 0.2 ml acetate buffer (pH 4.8, 0.1 mol/l). After addition of 5 μl of a 1 mmol/l aqueous DOTA-PESIN solution, the mixture was kept for 10 min at 90°C. The uncomplexed ^{68}Ga was separated by adsorption onto a SepPak C₁₈ cartridge that was equilibrated with 0.1 mol/l acetate buffer (pH 6.2), whereas [^{68}Ga]-DOTA-PESIN was eluted with 1.5 ml ethanol. After evaporation of the organic solvent, the radiotracer was redissolved in 0.01 mol/l phosphate-buffered saline (pH 7.4) containing 0.5 mg/ml human serum albumin. The preparations were checked for bound and free ^{68}Ga by paper chromatography using Whatman No.1 and methanol/0.01 mol/l acetate buffer (pH 6.2) at a ratio of 55:45. ^{68}Ge contamination of the [^{68}Ga]-DOTA-PESIN preparation was determined by gamma counting after a decay period of ≥ 30 h.

Binding affinity and receptor subtype profile

Using [^{125}I -D-Tyr⁴]BN as a GRP-R preferring ligand, the IC₅₀ values of [$^{\text{nat}}\text{Ga}/^{\text{nat}}\text{Lu}$]-DOTA-PESIN were measured in competitive binding experiments performed with increasing concentrations of $^{\text{nat}}\text{Ga}/^{\text{nat}}\text{Lu}$ -labelled peptides in successive tissue sections containing tumours expressing GRP-R [29].

The binding affinity profile for the three BN receptor subtypes was determined as described in detail previously [11, 30]. Human tumours were selected that have previously been shown to express predominantly one single BN receptor subtype. IC₅₀ values were determined in competitive binding experiments performed with increasing amounts of $^{\text{nat}}\text{Ga}/^{\text{nat}}\text{Lu}$ -DOTA-PESIN using [^{125}I -D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]BN(6–14) as universal radioligand.

Cell culture

PC-3 cells (ECACC, Wiltshire SP4 OJG, UK) were cultured in DMEM. DMEM was supplemented with vitamins,

essential and non-essential amino acids, L-glutamine, antibiotics (penicillin/streptomycin), fungicide [amphotericin B (Fungizone)] and 10% FCS.

Internalisation and efflux studies

Internalisation and externalisation experiments were performed in six-well plates as described previously [17]. Briefly, approximately 3 kBq (0.25 pmol) of [$^{177}\text{Lu}/^{67}\text{Ga}$]-DOTA-PESIN was added to PC-3 cell-containing incubation medium ($0.5\text{--}1 \times 10^6$ cells per well) and incubated at 37°C in a 5% CO_2 environment at different time points (0.5, 1, 2, 4 and 6 h). A large excess of [Ga^{III}]-DOTA-PESIN (0.4 $\mu\text{mol/l}$, 150 μl) was used to determine non-specific internalisation. At each time point, the internalisation was stopped by removal of the medium followed by washing the cells with ice-cold solution composed of 0.9% NaCl/0.01 mol/l Na_2HPO_4 /0.01 mol/l KH_2PO_4 (pH 7.2). Cells were then treated (twice) with glycine buffer (0.05 mol/l glycine solution, pH adjusted to 2.8 with 1 mol/l HCl) for 5 min to distinguish between cell surface-bound (acid buffer removable) and internalised (acid-resistant) radioligand. Finally, cells were detached from the plates by incubation with 1 mol/l NaOH for 10 min at 37°C, and the radioactivity was measured in a gamma counter. The percentage of added activity per million cells (% of total) was calculated for internalisation at each time point.

For externalisation studies, PC-3 cells were allowed to internalise [$^{177}\text{Lu}/^{67}\text{Ga}$]-DOTA-PESIN for 2 h; they were then exposed to an acid wash, as described in the internalisation experiments, to dissociate the cell surface-bound radioligand. Then 1 ml of culture medium (1% FCS) was added to each well, the cells were incubated at 37°C in a 5% CO_2 environment and the externalisation of the cell-incorporated radioactivity was studied at different times. The culture medium was collected and measured for radioactivity. The percentage of total internalised radiotracer was calculated for efflux.

Biodistribution experiments in PC-3 tumour-bearing nude mice

After a slight anaesthesia with isoflurane in an air/oxygen mixture, athymic nude female mice (Harlan, the Netherlands) were implanted subcutaneously on the right flank with about 10 million PC-3 tumour cells, which were freshly expanded in 100 μl sterilised PBS solution.

Seven to ten days after inoculation the tumours weighed 60–130 mg and the mice were injected via the tail vein with 10 pmol radiolabelled peptide (about 0.12 MBq [^{67}Ga]-DOTA-PESIN), diluted in 0.9% NaCl (0.1% BSA, pH 7.4, total injected volume 100 μl). For the determination of non-

specific uptake in the tumour and receptor-positive organs, a group of four animals was injected with a mixture of 10 pmol radiolabelled peptide/50 μg [Ga^{III}]-DOTA-PESIN in 0.9% NaCl solution (injected volume, 150 μl). To study the biodistribution of [^{67}Ga]-DOTA-PESIN, mice were sacrificed at 1, 4 and 24 h post injection; the organs of interest were collected, rinsed of excess blood, blotted, weighed and counted in a γ -counter. The percentage of injected activity per gram (%IA/g) was calculated for each tissue. The total counts injected per animal were determined by extrapolation from counts of an aliquot taken from the injected solution as a standard. For the biodistribution study of [^{177}Lu]-DOTA-PESIN, mice were sacrificed at 4, 24, 48 and 72 h post injection.

To study the influence of lysine, 15 mg lysine was co-injected with 10 pmol [^{67}Ga]-DOTA-PESIN in 150 μl , and the animals were sacrificed at 4 h post injection.

Biodistribution studies with [^{67}Ga]-DOTA-PESIN were also performed at the German Cancer Research Centre under conditions of PET imaging with [^{68}Ga]-DOTA-PESIN. Six- to 8-week-old female Swiss CD1 *nu/nu* mice were inoculated with a PC-3 tumour in the right flank. Eleven to thirteen days later, tumours weighing 70–175 mg were ready for experiment. Fifteen pmol (0.61 MBq) [^{67}Ga]-DOTA-PESIN was injected into the lateral tail vein. Mice were sacrificed at 1 h p.i. and organs of interest collected for measurement as described previously [31].

PET imaging with [^{68}Ga]-DOTA-PESIN

PET imaging was performed on a Siemens ECAT EXACT HR⁺ scanner (Siemens/CTI, Knoxville, TN, USA), and PET data were acquired in 3D mode and reconstructed iteratively with a full 3D algorithm from a 256×256 matrix for viewing transaxial, coronal and sagittal slices of 0.57 mm thickness. Pixel size was 1.14 mm and transaxial resolution obtained was 2.8 mm. For imaging studies, an awake female CD1 *nu/nu* mouse that was inoculated with a PC-3 tumour on the right flank was lightly restrained and injected with 1.94 MBq, 15 pmol [^{68}Ga]-DOTA-PESIN via a lateral tail vein. One hour later the mouse was anaesthetised with isoflurane in an air/oxygen mixture and sacrificed, then positioned in the tomograph symmetrically in the centre of the field of view. Acquisition of PET data was obtained with a 30-min emission and a 10-min transmission scan.

Scintigraphy with [^{177}Lu]-DOTA-PESIN

Scintigraphic imaging of PC-3 tumour xenografts was performed on a PRISM 2000 XP camera. Two mice were inoculated with a PC-3 tumour on the right hind leg. After 16 days the tumours had grown enough for imaging. Both

animals were lightly restrained and injected with 13.5 MBq, 0.32 nmol [^{177}Lu]-DOTA-PESIN via a lateral tail vein; one of them was co-injected with 50 μg [Ga^{III}]-DOTA-PESIN. Four hours after injection, the animals were anaesthetised with isoflurane in an air/oxygen mixture and positioned on the head of the camera symmetrically in the centre of the field of view while anaesthesia was monitored by observing the breath frequency. Scans were taken 15 min after anaesthesia at 4, 24, 48 and 72 h post injection.

All animal experiments were performed in compliance with the Swiss regulations for animal treatment (Bundesamt für Veterinärwesen, approval no. 789), or with the German laws for the protection of animals.

Statistical analysis

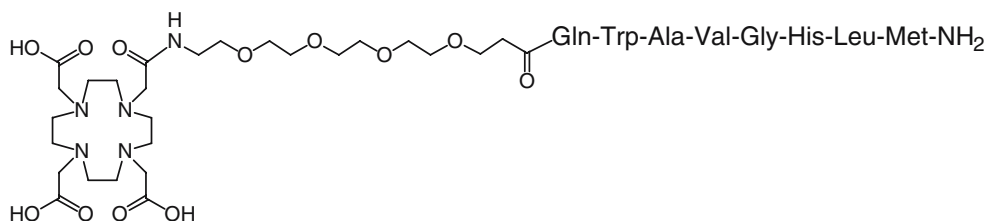
Data are expressed as mean \pm SD, which were calculated on Microsoft Excel. Student's *t* test (Origin 6, Microcal Software, Inc., Northampton, MA) was used to determine statistical significance at the 95% confidence level with $p < 0.05$ being considered significantly different.

Results

Synthesis and radiolabelling

DOTA-PESIN (Fig. 1) was synthesised using Fmoc strategy, affording yields of 45% based on the removal of the first Fmoc group; the purity analysed by HPLC was $\geq 97\%$. DOTA-PESIN and [Ga^{III} / Lu^{III}]-DOTA-PESIN were characterised by electrospray and MALDI TOFF mass spectrometry. Radiolabelled DOTA-PESIN was prepared by incubation with $^{67/68}\text{GaCl}_3$ or $^{177}\text{LuCl}_3$ at elevated temperature. The labelling yield of [$^{67/68}\text{Ga}$]-DOTA-PESIN was $\geq 98\%$ at specific activities of $>129 \text{ GBq } \mu\text{mol}^{-1}$ for ^{68}Ga and $>12 \text{ GBq } \mu\text{mol}^{-1}$ for ^{67}Ga , respectively. The radiolytic oxidation of methionine in [^{177}Lu]-DOTA-PESIN was about 2% when the specific activity was $\leq 11.6 \text{ GBq } \mu\text{mol}^{-1}$ and reached 26% at $60 \text{ GBq } \mu\text{mol}^{-1}$. With optimisation of the labelling conditions, labelling yields of $\geq 95\%$ of [^{177}Lu]-DOTA-PESIN were achieved at a specific activity of $>85 \text{ GBq } \mu\text{mol}^{-1}$ in the presence of 400 μg methionine per 370 MBq ^{177}Lu .

Fig. 1 Structural formula of DOTA-PESIN



Receptor binding affinity and profile

Table 1 shows the GRP-R binding affinities of DOTA-PESIN and its respective metallopeptides to human cancer tissue overexpressing GRP-R, as determined by a competitive binding assay using [^{125}I -Tyr 4]BN as radioligand. On the human GRP-R, the IC_{50} values were $9.5 \pm 3.4 \text{ nmol/l}$ for DOTA-PESIN and $6.1 \pm 3.0 \text{ nmol/l}$ and $6.6 \pm 0.1 \text{ nmol/l}$ for the Lu^{III} - and Ga^{III} -complexed peptides, respectively. There was no significant difference on the mouse GRP-R.

[Ga^{III}]-DOTA-PESIN and [Lu^{III}]-DOTA-PESIN were also studied with respect to the BN receptor subtype profile using human cancer tissue shown to express predominantly a single BN receptor subtype. Using [^{125}I -D-Tyr 6 , β -Ala 11 , Phe 13 , Nle 14]BN(6–14) as radioligand, the binding studies showed good binding affinities to NMB-R and GRP-R, and no affinity to BB3-R.

Internalisation and efflux studies

Internalisation of both radiopeptides was followed for 6 h. Both ^{177}Lu - and ^{67}Ga -labelled DOTA-PESIN showed specific and time-dependent cell uptake, reaching $39.1 \pm 1.1\%$ and $43.7 \pm 1.8\%$ of the total amount added to the medium, respectively, after 6 h of incubation at 37°C (Fig. 2). Within the first 2 h of incubation [^{177}Lu]-DOTA-PESIN internalised somewhat faster than [^{67}Ga]-DOTA-PESIN but after 4 h there was no statistical difference. The efflux kinetics (Fig. 3) showed a cellular retention of $52.5 \pm 1.6\%$ of the total internalised activity 20 h after start of efflux for both radiopeptides.

Animal biodistribution studies

Biodistribution studies using the $^{67}\text{Ga}/^{177}\text{Lu}$ -labelled peptides were performed with athymic nude female mice bearing the PC-3 human prostate tumour; the results are presented in Tables 2 and 3 as percentage of injected activity per gram of tissue (%IA/g).

According to Table 2, [^{67}Ga]-DOTA-PESIN and [^{177}Lu]-DOTA-PESIN (data in parentheses in the following) displayed rapid blood clearance from the PC-3-bearing mice, with $0.47 \pm 0.06 \text{ \%IA/g}$ at 1 h and 0.11 ± 0.04 (0.04 ± 0.01) \%IA/g at 4 h post injection. Both radiotracers were

Table 1 IC₅₀ values for metallated DOTA-PESIN when competed with [¹²⁵I-Tyr⁴]BN against the human and mouse GRP-R and affinity profiles using [¹²⁵I-Tyr⁶, β-Ala¹¹, Phe¹³, Nie¹⁴]BN(6–14)

Compound	Human GRP-R	Mouse GRP-R	BN receptor subtypes		
			NMB-R	GRP-R	BB3-R
DOTA-PESIN	9.5±3.4 (3)	ND	ND	ND	ND
[Lu ^{III}]-DOTA-PESIN	6.1±3.0 (3)	ND	15.0±4.0 (2)	8.3±1.7 (2)	>1,000 (2)
[Ga ^{III}]-DOTA-PESIN	6.6±0.1 (3)	7.0±1.2 (3)	12.5±0.5 (2)	10.0±0.0 (2)	>1,000 (2)

IC₅₀ values (nmol/l±SE) are in triplicates. Numbers of independent studies are listed in parentheses
 ND not determined

quickly washed out from the GRP-R-negative tissues except the kidneys. [⁶⁷Ga/¹⁷⁷Lu]-DOTA-PESIN also showed high uptake in the human prostate tumour xenograft and in the mouse GRP-R-positive organs, e.g. at 4 h the tumour uptake was 8.77±1.88 (7.46±1.64) %IA/g and the pancreas uptake 43.8±6.9 (39.0±4.9) %IA/g. In vivo competition experiments (Table 2) involving co-injection of 50 µg [Ga^{III}]-DOTA-PESIN with [⁶⁷Ga]-DOTA-PESIN resulted in a >94% reduction of uptake in the tumour and also in a reduction of uptake in other GRP-R-positive organs, e.g. >95% in the pancreas and 97% in the pituitary, 83–85% in the adrenals, spleen and bowel, and 78% in the stomach, showing specific receptor-mediated uptake in these tissues. The injection of the blocking dose had no significant influence on the uptake in other non-target organs whereas it led to a somewhat decreased kidney uptake. Co-injection of lysine did not reduce the kidney uptake, and the ratio between tumour and kidney remained almost unchanged. The impact of the blocking dose on biodistribution of [¹⁷⁷Lu]-DOTA-PESIN can be seen from the scans shown in Fig. 6.

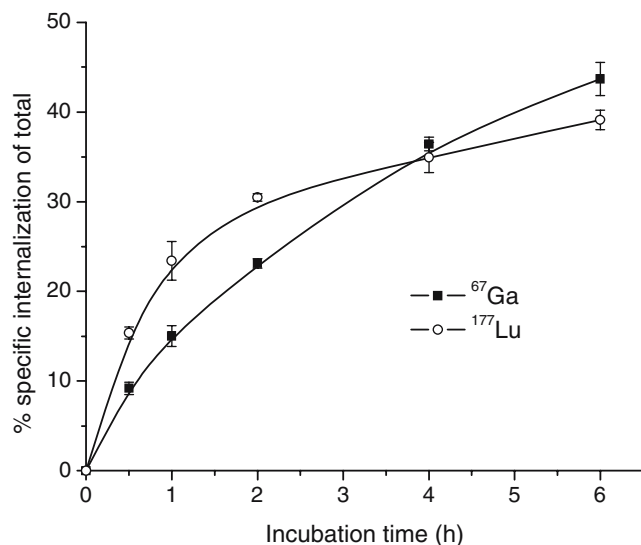


Fig. 2 Internalisation of ⁶⁷Ga-labelled (■) and ¹⁷⁷Lu-labelled (○) DOTA-PESIN into PC-3 cells. Data are from two independent experiments with triplicates in each experiment and are expressed as % specific internalisation of total activity added to the medium

Due to the rapid clearance from the body, high tumour-to-background ratios were found (Table 3). These ratios increased with time. For instance, the ratios of [⁶⁷Ga]-DOTA-PESIN between tumour and blood (muscle) varied from 31 (62) to 211 (237) during 1 h to 24 h post injection; for [¹⁷⁷Lu]-DOTA-PESIN, the respective ratios were 201 (147) at 4 h, 215 (387) at 24 h and 715 (486) at 72 h post injection. Both radiotracers also displayed high tumour-to-kidney and tumour-to-liver ratios (in parentheses): 2.4–2.6 (22–23) for the ⁶⁷Ga-labelled and 1.7–3.6 (36–52) for the ¹⁷⁷Lu-labelled peptide.

The pharmacokinetics (Fig. 4) of [⁶⁷Ga]-DOTA-PESIN shows the potential to visualise the tumour very early owing to the high initial accumulation, which reached 14.8±2.4 % IA/g at 1 h p.i. (9.40±1.39 %IA/g with an injected dose of 15 pmol and a different mouse strain), decreased to 8.77±1.88 %IA/g at 4 h and was still 6.76±0.29 %IA/g at 24 h. [¹⁷⁷Lu]-DOTA-PESIN also displayed a high uptake in the tumour and a relatively slow washout: 7.46±1.64 %IA/g at 4 h, 5.81±0.40 %IA/g at 24 h, 4.05±0.23 %IA/g at

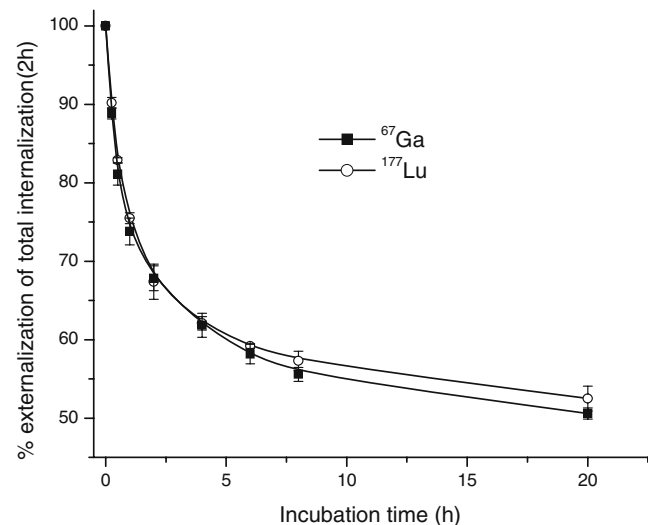


Fig. 3 Externalisation of ⁶⁷Ga-labelled (■) and ¹⁷⁷Lu-labelled (○) DOTA-PESIN from PC-3 cells. Cells were allowed to internalise for 2 h. Data are from two independent experiments with triplicates in each experiment and are expressed as % specifically externalised of total activity internalised added to the medium

Table 2 Biodistribution analysis (%IA/g \pm SD) of ^{67}Ga and ^{177}Lu -labelled DOTA-PESIN in PC-3 tumour-bearing nude mice

Site	$^{67}\text{Ga}^a$	$^{67}\text{Ga}^b$				$^{177}\text{Lu}^b$
	1 h	1 h	4 h	4 h blocked ^c	4 h lysine ^d	4 h
Blood	0.556 \pm 0.120	0.471 \pm 0.060	0.108 \pm 0.041	0.103 \pm 0.029	0.235 \pm 0.012	0.037 \pm 0.011
Muscle	0.160 \pm 0.046	0.243 \pm 0.170	0.042 \pm 0.008	0.043 \pm 0.004	0.068 \pm 0.014	0.051 \pm 0.015
Adrenals	NC	20.6 \pm 2.1	15.8 \pm 3.2	2.80 \pm 2.59	16.1 \pm 2.4	15.3 \pm 3.4
Pancreas	47.7 \pm 4.7	65.3 \pm 8.7	43.8 \pm 6.9	2.26 \pm 2.00	54.8 \pm 8.3	39.0 \pm 4.9
Spleen	2.62 \pm 0.28	2.67 \pm 0.47	2.25 \pm 0.16	0.48 \pm 0.05	2.36 \pm 0.10	2.55 \pm 0.55
Kidney	4.27 \pm 0.66	6.27 \pm 0.94	3.36 \pm 0.47	2.09 \pm 0.27	4.67 \pm 0.97	4.67 \pm 0.78
Stomach	NC	4.77 \pm 0.65	1.72 \pm 0.86	0.39 \pm 0.28	2.39 \pm 0.21	1.68 \pm 0.43
Bowel	7.23 \pm 0.66	7.41 \pm 0.79	3.65 \pm 1.55	0.57 \pm 0.47	5.75 \pm 1.21	3.77 \pm 1.30
Liver	0.50 \pm 0.10	0.64 \pm 0.15	0.41 \pm 0.06	0.44 \pm 0.15	0.37 \pm 0.03	0.21 \pm 0.04
Lung	0.60 \pm 0.12	0.69 \pm 0.16	0.41 \pm 0.16	0.19 \pm 0.05	0.22 \pm 0.03	0.10 \pm 0.04
Heart	NC	0.31 \pm 0.08	0.06 \pm 0.01	0.06 \pm 0.01	0.12 \pm 0.01	0.06 \pm 0.01
Bone	0.35 \pm 0.06	0.60 \pm 0.20	0.40 \pm 0.14	0.22 \pm 0.10	0.62 \pm 0.07	0.48 \pm 0.06
Pituitary	NC	18.7 \pm 9.8	3.35 \pm 1.62	0.09 \pm 0.08	3.07 \pm 0.77	3.44 \pm 0.53
Tumour	9.40 \pm 1.39	14.8 \pm 2.5	8.77 \pm 1.88	0.52 \pm 0.21	8.87 \pm 1.1	7.46 \pm 1.63

Results are the mean of groups of four to eight mice

NC not collected

^a PC-3 tumour-bearing CD nude mice were used for the biodistribution of 15 pmol (0.61 MBq) [^{67}Ga]-DOTA-PESIN

^b 10 pmol [$^{67}\text{Ga}/^{177}\text{Lu}$]-DOTA-PESIN was injected to PC-3 tumour-bearing athymic nude mice

^c Blocked by co-injection of 50 μg [Ga^{III}]-DOTA-PESIN

^d Co-injection of 15 mg lysine per mouse

48 h and 2.43 \pm 0.12 %IA/g at 72 h. In the pancreas, [^{177}Lu]-DOTA-PESIN was released faster than the ^{67}Ga -labelled peptide. In addition, the release of [^{177}Lu]-DOTA-PESIN from the pancreas and kidneys was faster than from the tumour, leading to an increasing tumour-to-organ ratio with time.

PET imaging

Iteratively reconstructed PET images of PC-3 tumour xenografts with [^{68}Ga]-DOTA-PESIN (1.94 MBq, 15 pmol) were performed at 1 h post injection (Fig. 5). The PC-3 tumour was located in the thoracic wall, and was perceptible with clear contrast from the adjacent background in the transaxial slices. Coronal slices distinctly show the tumour and kidneys. Sagittal slices demonstrate that [^{68}Ga]-DOTA-PESIN accumulates predomi-

nantly in the PC-3 tumour, pancreas and kidneys. The results of direct tissue counting and of PET imaging were consistent.

Scintigraphy

Figure 6 shows the scintigraphic imaging of PC-3 tumour xenografts with [^{177}Lu]-DOTA-PESIN (left: unblocked; right: blocked with 50 μg [Ga^{III}]-DOTA-PESIN). Imaging was performed at 4, 24, 48 and 72 h after injection. In the blocked mouse the tumour could not be visualised and only the kidneys were visible, whereas the PC-3 tumour of the non-blocked mouse was visualised with clear contrast from the adjacent background at all time points. Prominent uptake was also observed in the pancreas and kidneys. The imaging kinetics showed that [^{177}Lu]-DOTA-PESIN was preferably retained in the tumour rather than in the

Table 3 Radioactivity ratios between PC-3 tumour and other organs of ^{67}Ga - and ^{177}Lu -labelled DOTA-PESIN in PC-3 tumour-bearing female athymic nude mice [$8 \geq n \geq 4$]

Time (h)	Blood		Muscle		Kidney		Liver	
	^{67}Ga	^{177}Lu	^{67}Ga	^{177}Lu	^{67}Ga	^{177}Lu	^{67}Ga	^{177}Lu
1	31		62		2.4		23	
4	81	201	209	147	2.6	1.7	22	36
24	211	215	237	387	2.4	2.5	23	43
48		476		488		3.1		52
Tumour-to-blocked tumour ratio (4 h)=16.9		715		486		3.6		48

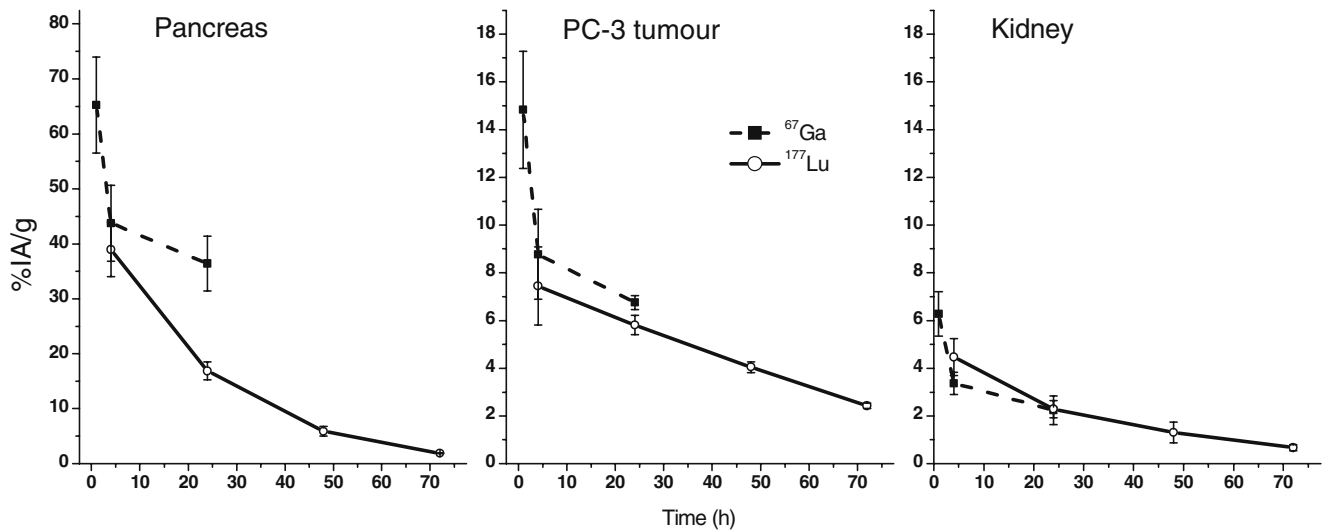


Fig. 4 Kinetics of ⁶⁷Ga-labelled (■) and ¹⁷⁷Lu-labelled (○) DOTA-PESIN in PC-3 tumour-bearing mice (*left*, pancreas; *middle*, PC-3 tumour; *right*, kidney)

pancreas and kidneys. These imaging results are consistent with biodistribution data (Fig. 4).

Discussion

We report on the development of a radiopeptide which can be used in SPECT (⁶⁷Ga), PET (⁶⁸Ga) and targeted radionuclide therapy (¹⁷⁷Lu) of BN receptor-positive tumours such as prostate and breast cancer or gastrointestinal stromal tumours. As part of a small library of peptides, which differ by the spacer between the chelate and the pharmacophoric peptide, DOTA-PEG₄-BN(7–14) appeared to have promising properties in terms of a relatively high tumour-to-kidney and tumour-to-liver ratio. Therefore we decided to study this peptide in depth after labelling with

⁶⁷Ga, ⁶⁸Ga and ¹⁷⁷Lu with the aim of introducing these radiopeptides into the clinic. DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was chosen as chelator since it forms M³⁺ complexes with high in vitro and in vivo stability [32]. PEG₄ (15-amino-4,7,10,13-tetraoxapentadecanoic acid) was chosen as spacer and pharmacokinetic modifier. Very long PEG-based spacers (>3,000 D) coupled to peptides or antibodies appear to decrease the binding affinity of these biomolecules to their receptors and to lower the rate of internalisation significantly, as shown by Rogers et al. using a [⁶⁴Cu]-DOTA-PEG-BN(7–14) derivative [33]. Smaller versions do not seem to have a sufficient influence on the pharmacokinetics and hydrophilicity [31, 33].

The presented peptide was synthesised on solid phase with an overall yield of >45%. ⁶⁸Ga labelling was straight-

Fig. 5 Iteratively reconstructed PET images of PC-3 tumour xenograft with [⁶⁸Ga]-DOTA-PESIN (1.94 MBq, 15 pmol) performed at 1 h post injection. The transaxial slice shows the PC-3 tumour (right flank) to be located in the thoracic wall. The coronal slice shows a clear distinction between tumour and kidneys. The sagittal slice demonstrates that [⁶⁸Ga]-DOTA-PESIN accumulates predominantly in the PC-3 tumour, pancreas and kidneys

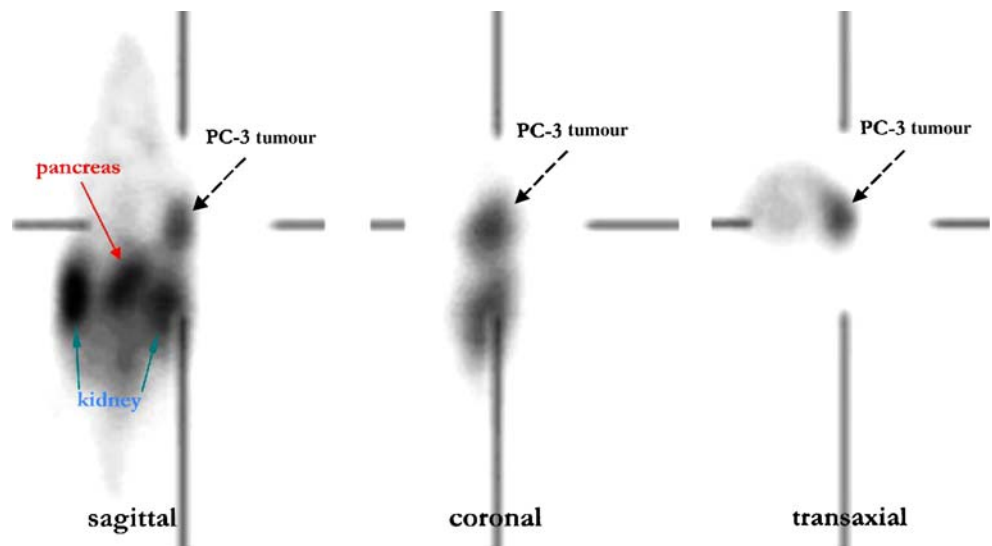
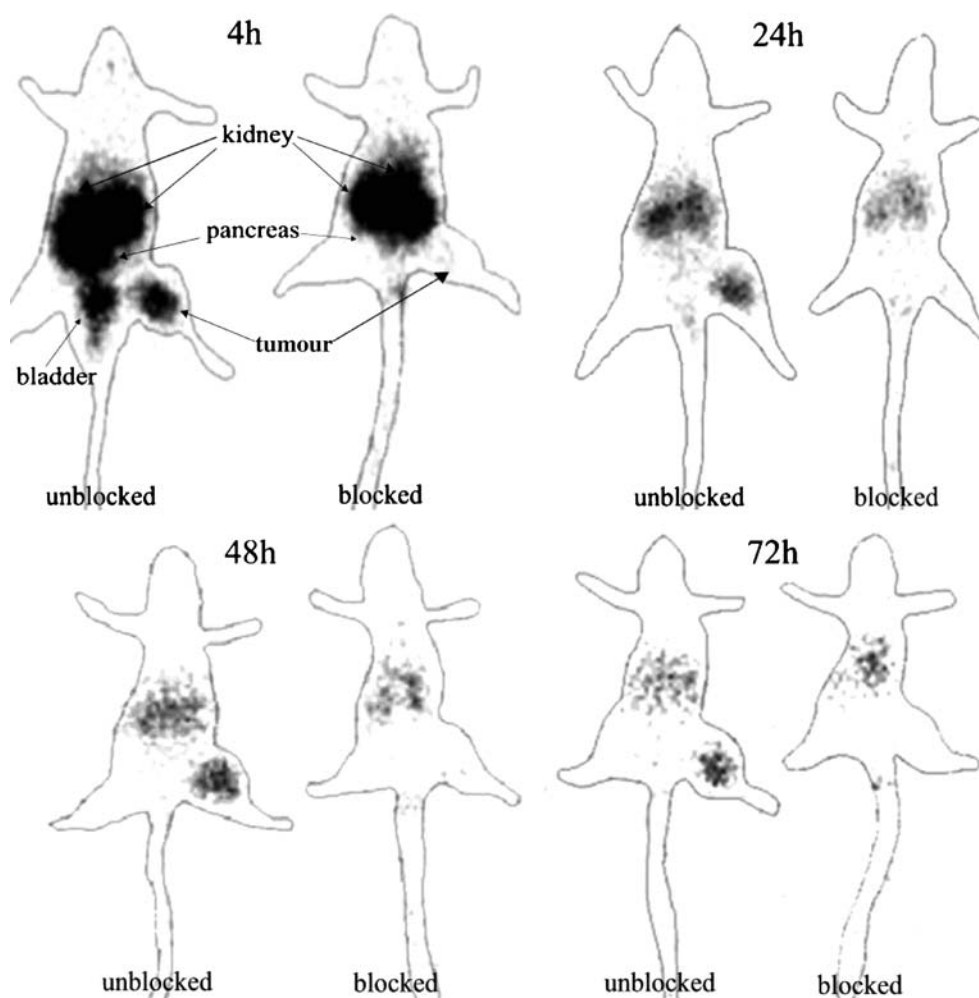


Fig. 6 Scintigraphic images of [^{177}Lu]-DOTA-PESIN in PC-3 tumour-bearing mice at 4, 24, 48 and 72 h post injection (13.5 MBq, blocked with 50 μg [^{67}Ga]-DOTA-PESIN. *Left*, unblocked; *right*, blocked)



forward and afforded high radiochemical yields at a reasonable specific activity of $\geq 130 \text{ GBq } \mu\text{mol}^{-1}$, which allowed production of good PET images at 1 h post injection. Sagittal slices clearly showed that [^{68}Ga]-DOTA-PESIN mainly localises the PC-3 tumour, the GRP receptor-positive pancreas and the kidneys.

Binding studies using the GRP receptor ligand [^{125}I -Tyr 4]BN revealed medium affinity of [^{67}Ga]-DOTA-PESIN and [^{177}Lu]-DOTA-PESIN to the human GRP receptor and no difference between human and mouse GRP receptors, at least for [^{67}Ga]-DOTA-PESIN. The affinity to the three human BN receptor subtypes using the universal radioligand [^{125}I -D-Tyr 6 , β -Ala 11 , Phe 13 , Nle 14]BN(6–14) showed that both metalloptides have good to moderate affinity to the GRP and the NMB receptor and no affinity to the BB3 receptor. The affinities are about ten times lower than those of truncated BN(7–14) and intact BN derivatives coupled to bifunctional tetra-amines, which show IC_{50} values between 0.2 and 2 nmol/l to the GRP receptor and, with one exception, also to the NMB receptor. The best of the $^{99\text{m}}\text{Tc}$ -labelled compounds have K_d values and IC_{50}

values below 1 nmol/l [19]. There was no significant difference in affinity between the peptides metallated with Ga^{3+} or Lu^{3+} . This is in contrast to DOTA-octreotide derivatives; these conjugates show a distinctly improved binding affinity when labelled with radiogallium [32, 34].

The rate of internalisation into PC-3 cells was similar for the two radiopeptides and somewhat more efficient than the internalisation of the panbombesin ligands studied previously in the same assay and under the same conditions [17]. This similarity does not reflect the distinct difference in binding affinity. The high rate of internalisation of the two radiopeptides indicates that they are agonists.

Efflux curves of [^{67}Ga]-DOTA-PESIN and [^{177}Lu]-DOTA-PESIN from the PC-3 cells after 2 h of internalisation are essentially identical and their half-lives are ≥ 20 h, which is much longer than those of the panbombesin derivatives. Efflux studies were also performed with [^{177}Lu -DOTA-Aoc]BN(7–14) but only up to 2 h [18]. Within this time period the two compounds behave similarly. [^{67}Ga -DOTA 0 -PEG $_2$, D-Tyr 6 , β -Ala 11 , Thi 13 , Nle 14]BN(7–14) and the corresponding ^{177}Lu -labelled radiopeptide externalise

with a somewhat different rate ($t_{1/2} (^{67}\text{Ga})=16.5\pm 2.4$ h; $t_{1/2} (^{177}\text{Lu})=12.8\pm 1.4$ h) [31]. A prolonged intracellular retention is of importance if long-lived radionuclides are to be used in therapy studies.

After administration of [$^{67}\text{Ga}/^{177}\text{Lu}$]-DOTA-PESIN, clearance from the circulation was fast and whole body clearance proceeded via the urinary system, unlike the positively charged $^{99\text{m}}\text{Tc}$ -N₄-labelled BN analogues, which are partly excreted via the hepatobiliary tract [19, 27]. Clearance from GRP receptor-negative tissues was also rapid except from the kidneys.

Initially, tumour uptake was very high (14.8% IA/g at 1 h), approaching the uptake of the best radiopeptides studied so far in the PC-3 tumour model [27]. This new radiopeptide has a high tumour-to-liver ratio which may make it even somewhat superior to the best $^{99\text{m}}\text{Tc}$ -labelled peptides [19]. The uptake in tumour, pancreas, intestine and pituitary was specific and receptor mediated, as shown by the co-injection of cold peptide, indicating that these organs are also GRP receptor positive. Unlike others [16, 35, 36] we also found a decrease in the kidney uptake when a peptide-blocking dose was administered, whereas a substantial reduction in kidney uptake upon blocking was also shown recently by use of an ^{18}F -labelled Lys³-BN derivative [37]. The fast background clearance of the radiogallium peptide renders [^{68}Ga]-DOTA-PESIN an ideal radiotracer for PET imaging. The relatively fast initial washout from the tumour may be explained by radioligand not being internalised and being metabolised during its presence in the tumour compartment.

The structural entities which confer the suitable pharmacokinetic properties to this molecule most likely reside in the PEG₄ spacer. Preliminary data using shorter and longer spacers indicate that PEG₄ has an optimal length. The lack of affinity to the BB3 receptor may be advantageous as this orphan receptor is expressed in the human pancreas.

Conclusion

Despite the relatively low binding affinity of [Lu^{III} , Ga^{III}]-DOTA-PESIN the corresponding radiopeptides show suitable pharmacokinetics in the PC-3 tumour-bearing mouse model, with high tumour uptake and relatively slow washout from the tumour. The radiopeptides show superior tumour-to-liver and suitable tumour-to-kidney ratios in comparison with our earlier published radiopeptides and even with the best $^{99\text{m}}\text{Tc}$ -labelled BN analogues. This renders the new peptides suitable for clinical studies.

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