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[Lys(DOTA)^4]BVD15, a novel and potent neuropeptide Y analog designed for Y_1 receptor-targeted breast tumor imaging

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ABSTRACT

We substituted a truncated neuropeptide Y (NPY) analog, [Pro^{30}, Tyr^{32}, Leu^{34}]NPY(28-36)NH$_2$ also called BVD15, at various positions with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7-10-tetraacetic acid) and evaluated the effect of the coupling position with the binding affinity for NPY Y_1 receptors (NPY1R). Our data suggest that [Lys(DOTA)^4]BVD15 (K_i = 63 ± 25 nM vs. K_i = 39 ± 34 nM for BVD15) is a potent NPY analog suitable for radiolabeling with metallo positron emitters for PET imaging of breast cancer.

Endocrine-sensitive tumors such as breast cancers overexpress several membrane and intracellular receptors that can be used for diagnostic imaging with radiopharmaceuticals. Neuropeptide Y (NPY) is a 36 amino acid neurohormonal amide peptide involved in feeding behavior, anxiety, memory and blood pressure regulation.[1] These actions are mediated by G-protein coupled receptors designated as Y_1, Y_2, Y_4, Y_5 and Y_6 (only in mice).[2] NPY Y_1 receptors (NPY1R) have been shown to be significantly overexpressed on a large proportion of breast cancers compared to Y_2 in normal breast tissues suggesting that neoplastic transformation can switch the NPY receptor expression from the Y_2 to the Y_1. The NPY1R is thus a prime candidate for receptor targeting with radiopharmaceuticals for highly specific tumor imaging by PET. The level of neuropeptide receptors is in part regulated by estradiol and progesterone in the hypothalamus.[3,4] Reubi et al. clearly demonstrated a significant overexpression of NPY1R at the surface of breast tumors in proportions ranging from 58% (45/77) to 85% (76/89),[5,6] whereas Y_2 receptors were present only focally in a small number of cases.[7] They also showed a dose-dependent inhibitory effect of NPY on the growth of breast cancer tumor cells, suggesting a functional significance for this receptor system. Amlal et al. recently showed the presence of NPY1R on the MCF-7 human breast cancer cell line.[8] Work done by Ruscica et al. on different human prostate cancer cell lines (LNCaP, DU145, and PC3) suggests that the growth of prostate cancer cells was regulated by the activation of the NPY1R via NPY.[9] The presence of NPY1R has also been shown in ovarian[7] and adrenal tumors[10] and some renal cell carcinomas and nephroblastomas.[11]

Only a few examples of radiometal labeled NPY analogs have been found in the literature.[12,13] The Beck-Sickinger group labeled modified NPY analogs with different radiometal/chelating agent combinations such as ^{99m}Tc/2-picolylamine-*N,N*-diacetic acid[12] or ^{111}In/DOTA[13] and obtained stable compounds with selective Y_1 and Y_2 binding. A 36 amino acid peptide, the Y_1 receptor selective [Lys(^{111}In/DOTA)^4, Phe^7, Pro^{34}]NPY, has shown promising characteristics for breast tumor diagnosis.[13] The aim of the present study was to develop potent truncated NPY analogs designed for Y_1 receptor-targeted breast tumor imaging. Several truncated NPY analogs targeting NPY1R have been developed.[14–17] We have selected BVD15 ([Pro^{30}, Tyr^{32}, Leu^{34}]NPY(28-36)-NH$_2$)[14] as a template peptide to design the novel radiotracer. Even though this peptide, developed by Daniels et al., also exhibits potent agonist activity at Y_2 and Y_4 receptors,[14] its affinity for the NPY1R is very high. The labeling of synthetic peptides is possible either during synthesis on solid support or in solution following synthesis of the peptide, depending on the radioactive isotope. For non-covalent radioactive isotopes such as metal ions like ^{68}Ga and ^{64}Cu to name a few,[13,18] modifications including appropriate chelators must be incorporated within the peptide sequence. As DOTA chelator is commercially available and forms stable complexes with various 3$^+$ charged radiometal such as Ga^{68}, it was first selected to optimize the design of our truncated NPY radiotracers. In this

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study, different sites of substitution on BVD15 were studied for the introduction of a DOTA chelating unit in order to maintain the peptide affinity to NPY1R (Fig. 1). N-Terminal elongation and truncation of the new peptide radiotracer designed for metal radiolabeling were also investigated.

Peptides were synthesized by continuous flow method on a Pioneer Peptide Synthesis System (PerSeptive Biosystems) with the Fmoc strategy.[19] Purity of the peptides was verified by analytical HPLC. Peptide identity was confirmed by MALDI-TOF MS. All peptides were prepared following the procedure described in note Ref. 19 with overall yields of 11–27% based on the substitution rate of the resin, determined photometrically from the amount of Fmoc chromophore released upon treatment of the resin with piperidine/DMF. The purity of the peptides was superior to 90% according to analytical HPLC. The peptide characteristics are summarized in Table 1.

Table 2
Affinities of [Lys(DOTA)^4]BVD15 for Y_1, Y_2, and Y_4 NPY receptors

NPY analogs	K_i[a] (nM)		
	Y_1	Y_2	Y_4
BVD15	5.0 ± 0.5[b] 39 ± 34	11.3 ± 3.8[b]	5.8 ± 0.9[b]
NPY, PYY or PP	3.1 ± 1.7	5.1 ± 1.4	0.36 ± 0.42
[Lys^4(DOTA)]BVD15	63 ± 25	No binding	No binding

[a] Affinities for Y_1 receptors were determined using ^{125}I-NPY, while those for Y_2 and Y_4 receptors were determined using ^{125}I-PYY and ^{125}I-PP, respectively. PYY and PP were used as control in cells expressing Y_2 and Y_4 receptors, respectively.
[b] Data are from Ref. 16.

Peptides were tested for receptor binding to various NPY receptor expressing cells; human breast carcinoma (MCF-7), human neuroblastoma (SK-N-BE2). The MCF-7 cells selectively express

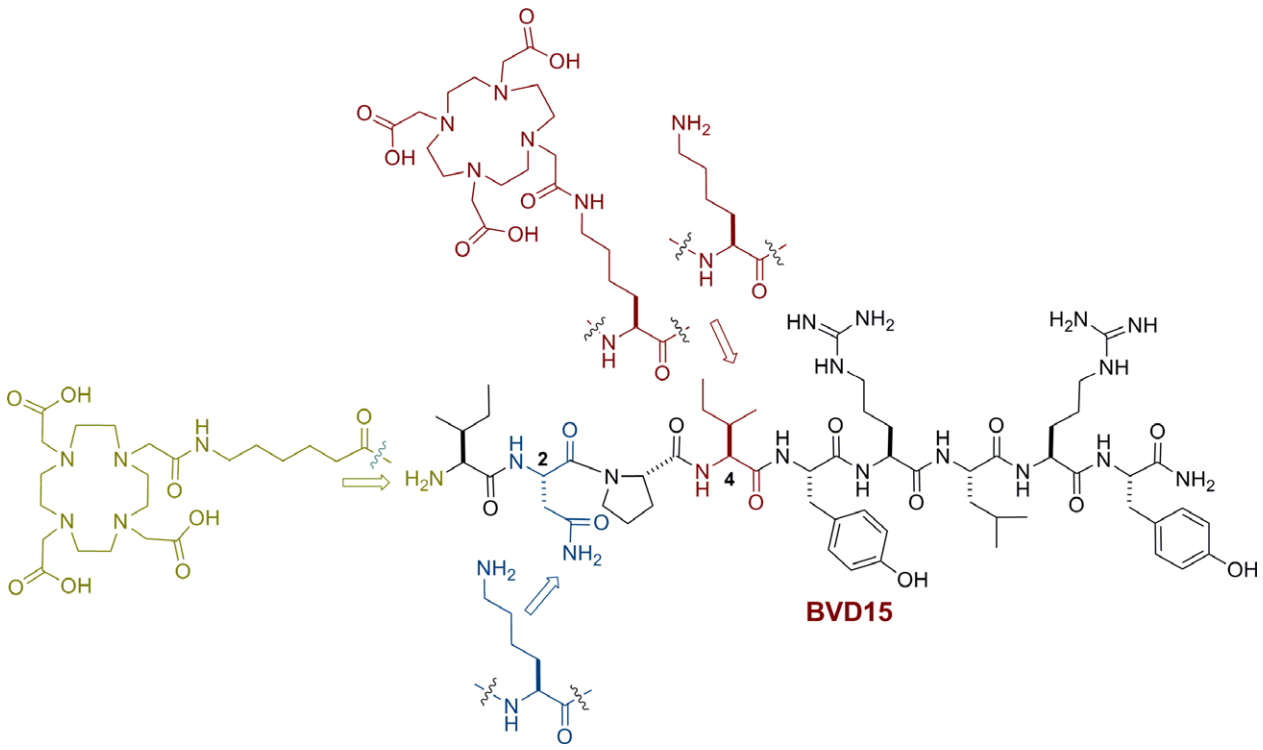

Figure 1. Structure of BVD15 and different structural modifications proposed.

Table 1
Analytical data for NPY analogs and affinities for NPY1R

List of NPY analogs	Mass		rt[b] (min)	Purity[c] (%)	K_i[d] (nM)
	Calcd	Found[a]			
NPY[e]					3.1 ± 1.7
BVD15	1206.4	1211.5 (M+5H)	12.3	99	39 ± 34
DOTA-Aoc-BVD15	1734.0	1738.7 (M+5H)	13.2	96	4507 ± 1753
[Lys^2]BVD15	1220.5	1223.6 (M+3H)	11.5	92	128 ± 64
[Lys^4]BVD15	1221.5	1225.3 (M+4H)	10.6	92	7 ± 3
[Lys(DOTA)^4]BVD15	1607.8	1607.8	10.7	90	63 ± 25
[Lys(Cu/DOTA)^4]BVD15	1669.4	1670 (M)	11.0	90	29 ± 15
Ac[Lys(DOTA)^4]BVD15	1649.9	1649.9 (M)	11.8	96	275 ± 99
Des-Ile^1[Lys(DOTA)^4]BVD15	1494.7	1494.1 (M)	10.5	92	No binding

[a] Masses were measured via MALDI mass spectrometry using Micromass Tof Spec 2F; at the time of these experiments, variations in the calibration were noticed, which explains slightly larger than usual variability in the results.
[b] HPLC retention time using agilent eclipse XDBC-18 column, 5 μm, 4.6 × 250 mm. The gradient used is composed of 0% for 5 min, and 0–100% CH_3CN (0.1% TFA) in H_2O (0.1% TFA) in 30 min.
[c] Purity was determined by HPLC analysis.
[d] Affinities for Y_1 receptors were determined using ^{125}I-NPY in MCF-7 cells.
[e] NPY was purchased from Calbiochem (Gibbstown, NJ, USA).

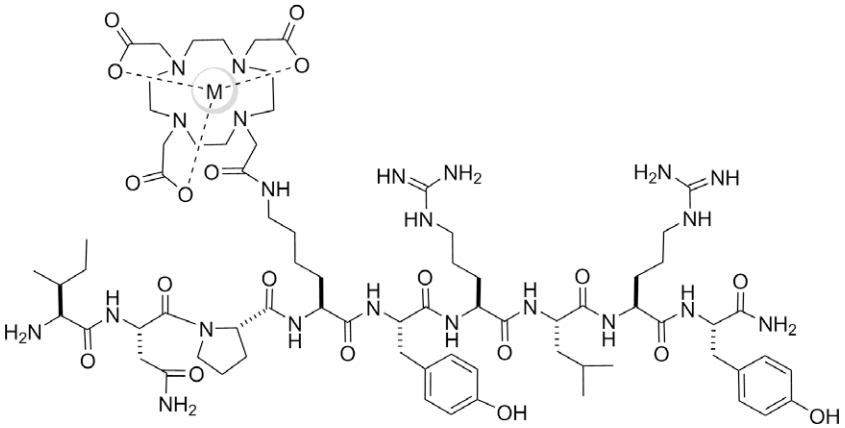

Figure 2. Structure of [Lys(M/DOTA)^4]BVD15 design for metal radiolabeling.

NPY1R, while SK-N-BE2 cells selectively express Y_2 receptors,[8] and HEK293 cells were transfected with the human Y_4 receptor cDNA.[20] MCF-7 and SK-N-BE2 cells were grown in high-glucose DMEM and EMEM/F12, respectively, both containing 10% FBS in the presence of penicillin/streptomycin, glutamine and amphotericin B. HEK293 cultured cells were transfected using Lipofectamine 2000. Competitive binding assays were repeated three times with at least triplicate data points at each concentration.[21]

Different sites for the introduction of the DOTA chelator were investigated. Variable NPY1R affinities were obtained with these molecules when tested in MCF-7 human breast cancer cells (Table 1). Based on previous work on the development of DOTA-Bombesin analogs,[18] we first explored the possibility to introduce the DOTA unit along with a flexible spacer, Aoc (amino octanoic acid), at the N-terminal part of the peptide. The affinity of DOTA-Aoc-BVD15 in MCF-7 cells had been shown to be significantly less than for BVD15, inhibition constant (K_i) values being 4507 ± 1753 nM and 39 ± 34 nM, respectively (Table 1). This result suggests that the introduction of a bulky group such as DOTA at the N-terminus of the peptide prevents the BVD15 peptide for interacting efficiently with the NPY1R. Studies performed in our laboratory have shown that poor affinity for NPY1R was obtained when BVD15 analog is substituted at its C-terminal position. For this reason, we did not investigate the possibility of introducing the DOTA unit at the C-terminus. Other sites for the introduction of the chelating unit on BVD15 were thus considered. Daniels et al. have shown that the Asn^2 and Ile^4 positions of BVD10, a methyl ester derivative of BVD15, can be replaced by other amino acids in the preparation of selective NPY1R antagonists.[14] We chose to substitute these amino acids by a lysine in order to ultimately attach the DOTA on the lysine side-chain of the peptide having the highest affinity for NPY1R. The highest K_i value was achieved with [Lys^2]BVD15, 128 ± 64 nM compared to 7 ± 3 nM for the [Lys^4]BVD15 analog (Table 1). From these results, it is clear that the amino acid substitution at position 4 was better tolerated for this truncated NPY analog. An excellent affinity for NPY1R was maintained even in a presence of a DOTA unit on the lysine side-chain at this position; the K_i value of [Lys(DOTA)^4]BVD15 being 63 ± 25 nM when tested in MCF-7 human breast cancer cell line. [Lys(Cu/DOTA)^4]BVD15 showed a similar affinity to Y_1 than the same peptide without copper, with a slightly lower K_i value. N-Terminal elongation and truncation of [Lys(DOTA)^4]BVD15 considerably affect the Y_1 affinity of the resulting peptides when tested in MCF-7 cells (Table 1). The K_i value of the acetylated peptide, Ac[Lys(DOTA)^4]BVD15, was more than four times higher than the original radiotracer. The truncated des-Ile analog did not bind to NPY1R suggesting that [Lys(DOTA)^4]BVD15 is the minimally sequence required for maintaining Y_1 affinity. As BVD15 has been reported to bind some extent to Y_2 and Y_4 receptors, the [Lys(DOTA)^4]BVD15 was examined for its subtype selectivity in SK-N-BE2 (Y_2) and Y_4 transiently transfected HEK293 cells (Table 2). The DOTA truncated NPY analog exhibited no activity to Y_2 and Y_4 receptors. This observation is in agreement with the lower affinities displayed by Cys^4-dimer of BVD15 for Y_2 and Y_4 receptors as reported by Daniels et al.[14]

In the present work, we described the design, the preparation and the in vitro activity of a new radiotracer designed for metal radiolabeling, [Lys(M/DOTA)^4]BVD15 (Fig. 2). Based on the in vitro results, it appears that [Lys(DOTA)^4]BVD15 is a potent and specific ligand for NPY1R. This work presents the first example of the shortest linear peptide prepared for NPY1R targeting. Further evaluation of the labeled [Lys(DOTA)^4]BVD15 with different metal radioisotopes as a PET breast cancer imaging agent is currently under investigation.

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19. *Peptide synthesis*: Using NovaSyn® TGR resin, a twofold excess of Fmoc-protected amino acids over resin substitution rate was utilized for coupling. Synthesis was performed using amine free DMF. Fmoc-protected amino acids were activated for coupling with an equimolar amount of HATU (2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate) and twofold excess of DIEA for 30 min. Fmoc deprotection was performed in 20% piperidine in DMF for 3 × 5 min. Peptides were deprotected and cleaved from the polymer support by treatment with a cocktail of TFA/H_2O/thioanisole (92:2:6) for 4 h. The resin was removed by filtration and washed with TFA. Combined filtrates were added dropwise to ethyl ether (10 mL of ether/mL of TFA). The precipitated crude peptides were centrifuged, and the ether solution was decanted. Crude peptides were dissolved in water and purified by flash chromatography on a Biotage SP4 system using FLASH+® C18 cartridge 25+M eluting with a linear gradient of 0–60% of acetonitrile in water for 10 volume of column with a flow rate of 25 mL/min and 0.1% TFA in all solvents. *Copper complexation*: The peptide was incubated at 50 °C for 1 h with an equimolar amount of $Cu(OAc)_2$ in 0.1 ammonium acetate buffer, pH 5.5.
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21. *Competition assays*: 30 μL of Lipofectamine 2000 combined with 470 μL OptiMEM medium was added to 500 μL OptiMEM medium containing 15 μg of human Y_4 receptor cDNA, which was previously inserted in expressing pcDNA3 vectors. The mixture was left at room temperature for 20 min, then added to a 150 mm dish containing HEK293 cells at 70% confluence and returned to the incubator. The medium was changed for OptiMEM medium 10% FBS after 3 h. Transiently transfected HEK293 cells were used 48 h after transfection. Competition assays were performed in 24 well plates, using MCF-7, SK-N-BE2 and HEK293 cells expressing the human Y_4 receptor for Y_1, Y_2, and Y_4 assays, respectively. The cells were cultured to near confluence. For the assay, the culture medium was replaced with reaction medium (RPMI medium, pH 7.4, containing 4.8 mg/mL HEPES and 2 mg/mL bovine serum albumin and amphotericin B) and ^{125}I-NPY (Perkin Elmer Life Sciences Canada, 81.4 TBq/mmol) or ^{125}I-peptide YY (^{125}I-PYY, Perkin Elmer Life Sciences Canada, 81.4 TBq/mmol), or ^{125}I-pancreatic polypeptide (125I-PP, Perkin Elmer Life Sciences Canada, 81.4 TBq/mmol) diluted to obtain 1.5–2 × 10^{-11} M per well. Increasing concentrations (10^{-5} to 10^{-13} M) of the NPY, PYY, or PP ligand of interest were added. Cells were then incubated at 37 °C with agitation for 40 min. After the incubation, the reaction medium was removed and the cells were washed three times with PBS at room temperature. The cells were harvested with trypsin and the bound ^{125}I-peptide was counted in a Cobra II gamma counter. Finally, data were analyzed with GRAPHPAD Software to determine IC_{50} value. The K_i was calculated from the IC_{50} using the equation of Cheng and Prusoff (Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099). The K_d values for the radioligands have been determined from experiments done under similar conditions; K_d = 5.2 × 10^{-10} M for [^{125}I-Lys^4]Neuropeptide Y, K_d = 4.3 × 10^{-10} M for ^{125}I-PYY, and K_d = 3.1 × 10^{-11} M for ^{125}I-PP.