Identification of a Suitable Peptidic Molecular Platform for the Development of NPY(Y1)R-Specific Imaging Agents

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NPY(Y1)R (neuropeptide Y receptor subtype 1) is an important target structure for tumor-specific imaging and therapy as this receptor subtype is overexpressed in very high density and incidence especially in human breast cancer. Targeting this receptor with radiolabeled truncated analogues of the endogenous ligand NPY (neuropeptide Y) has, however, not yet resulted in satisfactory imaging results when using positron emission tomography (PET). This can be attributed to the limited stability of these PET imaging agents caused by their fast proteolytic degradation. Although highly promising NPY analogues were developed, their stability has only been investigated in very few cases. In this systematical work, we comparatively determined the stability of the five most promising truncated analogues of NPY that were developed over the last years, showing the highest receptor affinities and subtype selectivities. The stability of the peptides was assessed in human serum as well as in a human liver microsomal stability assay; these gave complementary results, thus demonstrating the necessity to perform both assays and not just conventional serum stability testing. Of the tested peptides, only [Lys (lauroyl)]2,Pro25,Lys(DOTA)16,Bip27,Leu30]NPY27–36 showed high stability against peptidase degradation; thus this is the best-suited truncated NPY analogue for the development of NPY(Y1) R-specific imaging agents.

Introduction

Neuropeptide Y (NPY) is a peptide hormone consisting of 36 amino acids and has several regulatory functions in human physiological as well as pathological conditions. It binds to four different G protein-coupled receptors in humans (NPY(Y1)R, NPY(Y2)R, NPY(Y3)R and NPY(Y4)R) and regulates appetite, hormone secretion, cardiovascular and immune system, bone homeostasis, stress response and anxiety.[1,2] Furthermore, it is also involved in pathological processes such as obesity, hypertension, atherosclerosis, stress and several mood disorders.[3]

Recently, it was also found to be associated with different malignancies such as breast, renal cell and ovarian carcinomas, neuro- and nephroblastomas and others.[3] Especially in breast cancer, NPY(Y1)R is a highly promising target structure for tumor-specific imaging and therapy as this receptor subtype is overexpressed in very high density and incidence[4,5] (85% in primary human breast carcinomas and 100% of lymph node metastases of receptor-positive primaries), whereas non-neoplastic breast tissue only expresses NPY(Y3)R.

Thus, many attempts were made within the last years to develop radiolabeled NPY(Y1)R-specific analogues of NPY for the sensitive and specific imaging of this malignancy.[6] For this purpose, the full-length peptide can in principle be used, but short peptide sequences are much easier to synthesize than long ones and usually show better radiolabeling results as well as improved in vivo pharmacokinetics.[7] Furthermore, the full-length peptide is a pan-receptor ligand, showing no receptor subtype selectivity. Hence, the vast majority of approaches aimed at the development of truncated versions of NPY, ideally showing a strong receptor subtype preference as well as a high affinity to the NPY(Y1)R. One challenge is that the peptide-receptor interaction was demonstrated to be highly sensitive to structural changes of the peptide. The C-terminal part of the peptide, NPY28–36, is the minimal core sequence for receptor binding and Arg33, Arg35 as well as the terminal Tyr36-amide are essential for NPY(Y1)R binding.[2,7,8] Furthermore, Thr32 and Gln34 were found to be crucial for receptor subtype preference.[2] These prerequisites limit the possibilities of peptide modification to obtain truncated NPY analogues exhibiting the mentioned properties and being applicable for tumor imaging.

Another, even greater challenge in NPY-based radiotracer development is the physiological degradation of the lead peptide NPY as well as its truncated analogues. This limited stability of physiological peptides is a common phenomenon under in vivo conditions and is mainly mediated by proteolytic enzymes occurring in blood, liver and kidney.[9,10] In case of NPY and its analogues, the degradation is mediated by a vast
number of peptidases,\textsuperscript{[11]} cleaving the peptide at the pharmacophoric Arg33 and Arg35 residues\textsuperscript{[12,13]} and many other positions of the peptide sequence.\textsuperscript{[5]} This intrinsically and considerably limits the stability of radiolabeled NPY analogues and thus impedes successful tumor imaging using this peptide family. Of the many developed NPY derivatives, only few were tested for stability and applicability for tumor imaging and when determined, the truncated peptides showed an overall low\textsuperscript{[14,15]} or significantly decreased stability compared to NPY.\textsuperscript{[12]} This low stability was attributed to the reduced number of amino acids resulting in a poorly defined secondary structure of these short peptides entailing a faster proteolytic degradation.\textsuperscript{[14]}

Nevertheless, many truncated NPY analogues – several of them exhibiting very good to excellent in vitro properties such as high NPY(Y\textsubscript{1}R) affinity and receptor subtype selectivity\textsuperscript{[12,14,15,17,18,19,20,21]} – were developed with the aim of finding a peptide sequence applicable as NPY(Y\textsubscript{1}R)-specific tumor-targeting agent, for instance for molecular imaging. However, respective radiolabeled analogues applied for in vivo tumor visualization using highly sensitive in vivo PET imaging were seldomly reported and showed suboptimal pharmacokinetics\textsuperscript{[15,22]} caused by the stability issues discussed above.

One possibility to stabilize radiolabeled peptides against metabolic degradation is to modify them chemically, thereby hampering enzymatic breakdown.\textsuperscript{[21]} An alternative to this sometimes laborious structural modification in order to achieve higher in vivo stabilities of radiopeptides was proposed recently and showed favorable imaging results and strongly increased tumor uptakes. This was coinjection of the radioligands with enzyme inhibitors, protecting the applied GRPR-, SSTR- and CCK2R-specific radiotracers against proteolytic degradation (GRPR: gastrin-releasing peptide receptor, SSTR: somatostatin receptor, CCK2R: cholecystokinin receptor subtype 2).\textsuperscript{[23,24]} Although this approach showed in part extremely valuable results, it has to be adapted to the respective peptidic radiotracer to be able to increase its metabolic stability, thus also entailing further optimization steps.\textsuperscript{[23,26]}

Unfortunately, no stability data are available for most of the developed NPY(Y\textsubscript{1}R)-specific ligands and no systematic study has been performed so far directly comparing their metabolic stabilities. As a high stability of the receptor ligands is however a prerequisite for in vivo target accumulation and thus tumor visualization, NPY analogues exhibiting the mandatory high metabolic stability are required. Thus, we synthesized and directly compared DOTA introduction in analogues of NPY were developed, exhibiting a linear, cyclic or dimeric peptide backbone in order to identify derivatives with high NPY(Y\textsubscript{1}R) targeting ability.\textsuperscript{[12,14,15,17,18,19,20,21,23,29]} Among these, several showed low affinities, NPY(Y\textsubscript{1}R) receptor subtype selectivities or stabilities. Of those remaining, we chose to directly compare five different peptide analogues regarding their metabolic stability (Figure 1), representing the most promising agents in terms of in vivo properties (NPY(Y\textsubscript{1}R) affinity, Table 1, and receptor subtype selectivity) within their respective group of linear, cyclic and truncated NPY analogues: i) linear [Pro\textsuperscript{10},Lys(DOTA)\textsuperscript{15},Tyr\textsuperscript{16},Leu\textsuperscript{18}]NPY\textsubscript{29-36} ([Lys(DOTA)]-BVD\textsubscript{19}; 1; DOTA = (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid), serving as reference compound with known low metabolic stability but high NPY(Y\textsubscript{1}R) affinity and receptor subtype selectivity,\textsuperscript{[16]} ii) [Pro\textsuperscript{10},Lys(DOTA)\textsuperscript{15},Bip\textsuperscript{19},Leu\textsuperscript{51}]NPY\textsubscript{28-36} (2), a variant of 1 comprising an artificial Bip (biphenylalanine) amino acid in position 32 instead of tyrosine,\textsuperscript{[12]} and Lys(DOTA) in position 31 for radiolabeling,\textsuperscript{[15]} iii) [Lys(laurol)\textsuperscript{27}Pro\textsuperscript{10},Lys(DOTA)\textsuperscript{15},Bip\textsuperscript{19},Leu\textsuperscript{51}]NPY\textsubscript{27-36} (3), a variant of 2 modified with an additional N\textsubscript{ε}-Lys-lauroyl in position 27 for full receptor agonism and high NPY(Y\textsubscript{1}R) affinity,\textsuperscript{[19]} also modified with Lys(DOTA) in position 31 for radiolabeling, iv) Ac(p-Cys\textsubscript{29},Lys(DOTA)\textsuperscript{31},Cys\textsubscript{34}]NPY\textsubscript{29-36} (YM-42454, 4), a cyclic truncated NPY\textsubscript{29-36} analogue developed for conformational fixation, resulting in high NPY(Y\textsubscript{1}R) affinity and receptor subtype selectivity,\textsuperscript{[20]} which was also modified with Lys(DOTA)\textsuperscript{31} for radiolabeling, and v) heterodimer [Pro\textsuperscript{20},Cys\textsubscript{28},Trp\textsubscript{32},Nle\textsuperscript{40}]NPY\textsubscript{28-36} ([Lys(DOTA)]-BVD\textsubscript{19}; 5), also displaying a high NPY(Y\textsubscript{1}R) affinity.\textsuperscript{[19]}

For DOTA introduction in analogues 3 and 4, being required for radiolabeling and comparative stability assessment, we did not modify the respective peptides N-terminally as the N terminus was shown to be important for receptor activation.\textsuperscript{[12,18]} Alternatively, the chelator can, for example, be conjugated to a side-chain functionality of an additionally introduced amino acid at the N terminus.\textsuperscript{[18]} However, as it was shown for BVD\textsubscript{19} that the DOTA conjugation least impedes peptide–receptor interaction when introduced in position 31 by using Lys(N\textsubscript{ε}-DOTA),\textsuperscript{[16]} we applied this molecular design, resulting in peptide precursors 2, 3 and 4. Derivatives 1 and 5 were already described as DOTA-modified agents.

Results and Discussion

Truncated NPY derivatives chosen for comparative stability assessment

Over the last two decades, nearly 80 truncated peptides analogues of NPY were developed, exhibiting a linear, cyclic or dimeric peptide backbone in order to identify derivatives with high NPY(Y\textsubscript{1}R) targeting ability.\textsuperscript{[12,14,15,17,18,19,20,21,23,29]} Among these, several showed low affinities, NPY(Y\textsubscript{1}R) receptor subtype selectivities or stabilities. Of those remaining, we chose to directly compare five different peptide analogues regarding their metabolic stability (Figure 1), representing the most promising agents in terms of in vivo properties (NPY(Y\textsubscript{1}R) affinity, Table 1, and receptor subtype selectivity) within their respective group of linear, cyclic and truncated NPY analogues: i) linear [Pro\textsuperscript{10},Lys(DOTA)\textsuperscript{15},Tyr\textsuperscript{16},Leu\textsuperscript{18}]NPY\textsubscript{29-36} ([Lys(DOTA)]-BVD\textsubscript{19}; 1; DOTA = (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid), serving as reference compound with known low metabolic stability but high NPY(Y\textsubscript{1}R) affinity and receptor subtype selectivity,\textsuperscript{[16]} ii) [Pro\textsuperscript{10},Lys(DOTA)\textsuperscript{15},Bip\textsuperscript{19},Leu\textsuperscript{51}]NPY\textsubscript{28-36} (2), a variant of 1 comprising an artificial Bip (biphenylalanine) amino acid in position 32 instead of tyrosine,\textsuperscript{[12]} and Lys(DOTA) in position 31 for radiolabeling,\textsuperscript{[15]} iii) [Lys(laurol)\textsuperscript{27}Pro\textsuperscript{10},Lys(DOTA)\textsuperscript{15},Bip\textsuperscript{19},Leu\textsuperscript{51}]NPY\textsubscript{27-36} (3), a variant of 2 modified with an additional N\textsubscript{ε}-Lys-lauroyl in position 27 for full receptor agonism and high NPY(Y\textsubscript{1}R) affinity,\textsuperscript{[19]} also modified with Lys(DOTA) in position 31 for radiolabeling, iv) Ac(p-Cys\textsubscript{29},Lys(DOTA)\textsuperscript{31},Cys\textsubscript{34}]NPY\textsubscript{29-36} (YM-42454, 4), a cyclic truncated NPY\textsubscript{29-36} analogue developed for conformational fixation, resulting in high NPY(Y\textsubscript{1}R) affinity and receptor subtype selectivity,\textsuperscript{[20]} which was also modified with Lys(DOTA)\textsuperscript{31} for radiolabeling, and v) heterodimer [Pro\textsuperscript{20},Cys\textsubscript{28},Trp\textsubscript{32},Nle\textsuperscript{40}]NPY\textsubscript{28-36} ([Lys(DOTA)]-BVD\textsubscript{19}; 5), also displaying a high NPY(Y\textsubscript{1}R) affinity.\textsuperscript{[19]}

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Figure 1. Structures of the investigated truncated and DOTA-modified NPY analogues 1–5. NPY-derived structure elements are highlighted in turquoise, pharmacophoric structural elements for NPY(Y9)R affinity of native peptide derivative. [a] Radiochemical yield. [b] Non-optimized molar activity. [c] In human serum. [d] In human liver microsomal assay. [e] NPY(Y9)R affinity of DOTA-modified peptide derivative. [f] NPY(Y9)R affinity of native peptide derivative.

Table 1. Summary of compound properties

| Compound | RCY(y) | A μmol [GBq/μmol] | log D | t1/2 [min] | t1/4 [min] | NPY(Y9)R affinity [nM] |
|----------|--------|------------------|-------|-------------|-------------|------------------------|
| 1(113Ga) | HR-ESI-MS (m/z) for [M + 4H]+: (calcd): 402.7305 (402.7314), [M + 3H]+: (calcd): 536.6383 (536.6393), [M + 2H]+: (calcd): 804.4537 (804.4550); MALDI-MS (m/z) for [M + H]+: (calcd): 1607.40 (1607.90), [M + Na]+: 1629.51 (1629.88), [M + K]+: 1645.50 (1645.86) | 99.4% | 21.2–23.1 | –3.61 ± 0.37 | 20 | > 100 | IC50: 7 ± 3[15] |
| 2(111Ga) | HR-ESI-MS (m/z) for [M + 4H]+: (calcd): 417.7397 (417.7405), [M + 3H]+: 556.6504 (556.6514); MALDI-MS (m/z) for [M + H]+: (calcd): 1667.83 (1667.94), [M + Na]+: 1689.90 (1689.92), [M + K]+: 1705.29 (1705.89) | 99.2% | 19.0–21.4 | –3.01 ± 0.13 | 65 | > 100 | IC50: 29.7 ± 6.8[12] |
| 3(111Ga) | HR-ESI-MS (m/z) for [M + 4H]+: (calcd): 495.2564 (495.3060), [M + 3H]+: 660.0712 (660.0721); MALDI-MS (m/z) for [M + H]+: (calcd): 1978.01 (1978.20), [M + Na]+: 1999.95 (2000.18), [M + K]+: 2016.17 (2016.16) | 98.7% | 18.9–21.0 | –0.48 ± 0.14 | 144 | > 100 | IC50: 1.6 ± 0.2[16] |
| 4(111Ga) | MALDI-MS (m/z) for [M + H]+: (calcd): 1467.57 (1467.72), [M + Na]+: 1489.56 (1489.70), [M + K]+: 1505.90 (1505.68) | – | – | – | – | – | KIC50: 47 ± 11[32] |
| 5(111Ga) | HR-ESI-MS (m/z) for [M + 6H]+: (calcd): 473.5914 (473.5921), [M + 5H]+: 568.1081 (568.1089); MALDI-MS (m/z) for [M + H]+: (calcd): 2836.28 (2836.51), [M + Na]+: 2858.78 (2858.50), [M + K]+: 2874.72 (2874.47) | 96.0% | 18.7–21.4 | –2.47 ± 0.18 | 67 | 6 | IC50: 13 ± 3[25] |

[a] Radiochemical yield. [b] Non-optimized molar activity. [c] In human serum. [d] In human liver microsomal assay. [e] NPY(Y9)R affinity of DOTA-modified peptide derivative. [f] NPY(Y9)R affinity of native peptide derivative.
Chemical synthesis of chelator-modified peptides 1–5

Peptides 1–3 could be synthesized by applying standard Fmoc-based solid phase peptide synthesis (SPPS) protocols, standard Fmoc-\textit{N\textsubscript{\textalpha}}-amino acids, Fmoc-L-Bip-\textit{OH} and Fmoc-L-Lys(lauroyal)-\textit{OH} and HBTU (2-(1H-Benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate) as coupling agent. To enable DOTA conjugation to the \textit{N\textepsilon} amino function of lysine, Fmoc-l-Lys(Mtt)-\textit{OH} was used during the synthesis of the linear peptide sequences. After mild acidic cleavage of the Mtt protecting group with 1.5 % TFA (trifluoroacetic acid) in CH\textsubscript{2}Cl\textsubscript{2}, DOTA-tris(\textit{t}Bu)ester was coupled using standard reaction conditions but PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate) activation and prolonged coupling times of 1 h.

Compound 4 was synthesized accordingly, using the Acm-protected cysteine derivatives Fmoc-d-Cys(Acm)-\textit{OH} and Fmoc-l-Cys(Acm)-\textit{OH} during peptide assembly, followed by acetylation of the N-terminus by using acetic anhydride and DIPEA (N,N-diisopropylethylamine) in DMF. Afterwards, the oxidative deprotection and simultaneous cyclization of the peptide was carried out using Tl(TFA\textsubscript{3}).

To obtain 5, a new synthetic strategy had to be established as the procedure described previously gave the product in insufficient yields of ~1 % and low purity of only 83 %. In the published protocol, both peptide monomers were synthesized according to a Boc-strategy and purified, then the S-Acm protecting groups were removed by using silver trifluoromethane sulfonate. The heterodimer was subsequently obtained by reacting both monomers in equimolar amounts under oxidative conditions in solution, forming the intramolecular \textit{S–S}-bond. Under these conditions, the main contaminants were the homodimers which could not be fully separated, resulting in the low product purity of 83 %. In a first attempt to obtain 5 in pure form, we synthesized both peptide monomers 6 and 7 using standard Fmoc-based SPPS protocols and Fmoc-Cys (Acm)-\textit{OH} as cysteine derivative during peptide synthesis (Scheme 1).

The non-DOTA-modified monomer was built on a highly acid-labile Sieber amide resin, giving the fully protected peptide 6 after mild acid cleavage whereas the other, DOTA-modified monomer 7 was synthesized on a standard rink amide resin. Fully protected monomer 6 was then added in solution to the DOTA-modified peptide 7 (still on resin) together with Tl(TFA\textsubscript{3}) to produce the peptide heterodimer on resin. After cleavage, only the intended heterodimer was expected to have formed. Unfortunately, this approach did not give the product 5 in sufficient yield as the formation of the dithiol bond showed to take place only to a very low extent, potentially due to the steric hindrance caused by the fully protected peptides.

Thus, another attempt was made using a different strategy, depicted in Scheme 2. First, we synthesized the deprotected asparagine-containing monomer 11 by standard Fmoc-based SPPS protocols. The DOTA-modified counterpart 8 was also obtained applying standard methods as described before, using Fmoc-Cys(STmp)-\textit{OH} instead of standard Fmoc-Cys(Trt)-\textit{OH}.

Scheme 1. Depiction of the synthetic route towards 5 used initially. It did not give the product in sufficient yield and purity.
during the synthesis of the linear peptide. The STmp-protecting group could be quantitatively removed under mild reductive conditions using 2-mercaptoethanol and N-methylmorpholine (NMM) in DMF within 15 minutes, yielding the free thiol intermediate 9 on solid support. This thiol could be modified with 2,2’-dithio-bis(5-nitropyridine) (DTNP), giving the heterodisulfide intermediate 10 which easily reacted chemoselectively with the thiol moiety in peptide 11, forming peptide heterodimer 5 and giving the pure product in at least moderate yield of 8% after HPLC purification.

\[ \text{Scheme 2. Depiction of the improved synthesis pathway that yielded 5 in pure form.} \]

\[ \text{Ga-Radiolabeling and determination of the log}_D \text{ of the peptide monomers [}^{68}\text{Ga}]_1–[}^{68}\text{Ga}]_4 \text{ and heterodimer [}^{68}\text{Ga}]_5 \]

The peptide monomers 1–4 and the heterodimer 5 were radiolabeled with \[^{68}\text{Ga}]^3^+ \text{ (obtained by fractioned elution of an IGG }^{68}\text{Ge/}^{68}\text{Ga generator system) in aqueous acetate-buffered solution. The radiolabeling reaction itself took place at } 99^\circ \text{C within reaction times of 10 minutes at pH 3.5–4.0. The }^{68}\text{Ga}-\text{labeled products [}^{68}\text{Ga}]_1–[}^{68}\text{Ga}]_3 \text{ and [}^{68}\text{Ga}]_5 \text{ could be obtained in high radiochemical yields and purities of 96–99% as well as non-optimized molar activities of 18.8–23.1 GBq/μmol, starting from 376.6–461.0 MBq of }^{68}\text{Ga}^3^+ \text{.} \]

In contrast, pure [}^{68}\text{Ga}]_4 \text{ could not be obtained under these standard conditions, as the formation of a significant number of side products occurred which increased over time. We first assumed that this side product formation could be a result of thermal degradation or radiolysis during radiolabeling. However, thermal degradation could be ruled out to be the reason for the observed fragmentation as the precursor showed to be stable at elevated temperature of 99°C. Additionally, the peak pattern during radiolabeling did not change when the reaction was conducted at lower temperature of 45°C (apart from the increased fraction of free \[^{68}\text{Ga}]^3^+ \text{ in the radiolabeling reaction solution at this temperature). Neither did the presence of a high excess of ascorbic acid (10 mg) result in considerably higher product homogeneity, thus also excluding radiolysis as the reason for the observed formation of side product. In order to rule out any involvement of the thiols of the intramolecular dithiol bond in side-product formation, we added the reductive agent TCEP (tris(2-carboxyethyl)phosphine) to the reaction mixture. This did not result in a homogeneously radiolabeled product either. Furthermore, as the formation of side products was only observed for the radiolabeled agent, but not the} \]

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precursor (as is evident from the radio-HPLC and corresponding UV-HPLC traces), the origin of the observed side products remains inconclusive. Therefore, we omitted further characterization and stability testing of [\(^{68}\text{Ga}\)]\(^{3+}\) due to its limited radiochemical stability.

In the following, the log\(_{D}\) values of the radiolabeled peptide monomers [\(^{68}\text{Ga}\)]\(^{1-34}\) and the heterodimer [\(^{68}\text{Ga}\)]\(^{5}\) were determined as the log\(_{D}\) can significantly impact the in vivo clearance pathway of the respective compound (renal vs. hepatobiliary clearance).\(^{31,32}\) The log\(_{D}\) values were determined from the distribution coefficient of the respective radiotracer between phosphate buffer at pH 7.4 and octan-1-ol and are given in Table 1. As expected, the log\(_{D}\) values varied significantly, increasing from the highly hydrophilic [\(^{68}\text{Ga}\)]\(^{1}\) (log\(_{D}\) of \(-3.61 \pm 0.37\)) to lipophilic [\(^{68}\text{Ga}\)]\(^{3}\) (log\(_{D}\) of \(-0.48 \pm 0.14\)) due to the modification of the peptide with the lipophilic Bip amino acid and the lauroyl residue. [\(^{68}\text{Ga}\)]\(^{5}\) also showed a rather hydrophilic character (log\(_{D}\) of \(-2.47 \pm 0.18\)).

### Determination of the stability of peptide monomers [\(^{68}\text{Ga}\)]\(^{1-34}\) and heterodimer [\(^{68}\text{Ga}\)]\(^{5}\) in human serum and human microsomal stability assay

As NPY was shown before to be mainly metabolized by proteolytic degradation in tissues associated with the nervous system as well as in serum,\(^{11}\) the stability of the radioligands [\(^{68}\text{Ga}\)]\(^{1-34}\) and [\(^{68}\text{Ga}\)]\(^{5}\) was first to be determined by a human serum stability assay. This assay provides a good measure for radiotracer stability during blood pool circulation. Additionally, the stability of the compounds against degradation by liver enzymes was determined by an assay with human liver microsomes as metabolic degradation of some of the radioligands can be caused by liver enzymes as well. Especially [\(^{68}\text{Ga}\)]\(^{3}\) is supposed to show a considerable in vivo uptake in the liver due to its high lipophilicity. The human liver microsomal stability assay allows the assessment of the intrinsic metabolic clearance of substances by the human hepatobiliary cytochrome P450 system including degradation by cytochrome P450, oxidoreductase, cytochrome b\(_{6}\), CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP4A, and FMO.

In the stability assay performed in human serum, the radiotracers showed the formation of different, mostly hydrophilic metabolites and significant differences regarding the rate of degradation by peptidases, resulting in strongly differing serum half-lives of the compounds (Figure 2, Table 1).

\(^{68}\text{Ga}\)-Lys\(^{3}\)(DOTA)-BVD15 ([\(^{68}\text{Ga}\)]\(^{1}\)) showed – as expected from literature data\(^{12}\) – a low stability in human serum with a half-life of only 20 minutes. The replacement of the Tyr\(^{32}\) amino acid by artificial Bip in [\(^{68}\text{Ga}\)]\(^{2}\) however resulted in a considerable stabilization against degradation and substantially prolonged serum half-life to 65 minutes. The additional N-terminal lauroylation in [\(^{68}\text{Ga}\)]\(^{3}\) further improved the serum stability of the radiopeptide significantly, demonstrating a half-life of 144 minutes. This radiopeptide half-life is absolutely sufficient for \(^{68}\text{Ga}\)-PET imaging purposes as PET scans using this nuclide are usually performed within the first 60 to 90 minutes post injection. The heterodimer [\(^{68}\text{Ga}\)]\(^{5}\) demonstrated a serum half-life of 67 minutes, being comparable to that of [\(^{68}\text{Ga}\)]\(^{2}\).

As extensively studied before, NPY undergoes a proteolytic degradation mediated by a plethora of different peptidases, being present in the central and peripheral nervous system, cerebrospinal fluids and serum.\(^{19}\) In serum, the mainly active peptidase for NPY degradation was shown to be dipeptidyl peptidase 4 (DP4), N-terminally cleaving the peptide to exclusively give NPY\(_{3-36}\) as the metabolite. Thus, DP4 cannot be the peptidase producing the degradation pattern observed in this study as the truncated peptides studied here lack the respective part of the NPY peptide being cleaved by this peptidase. Apart from DP4, also C-terminal angiotensin-converting enzyme (ACE)-mediated degradation was shown to take place in serum, giving metabolite NPY\(_{1-34}\) being thus more relevant for the observed enzymatic degradation of the truncated NPY analogues studied here. Further proteins which could be involved in the metabolism observed here are cathepsins, plasma Kallikrein and plasmin.\(^{36}\) The known cleavage pattern of truncated NPY\(_{28-36}\) by these peptidases is depicted in Figure 3.

In the liver microsomal stability assay (using liver microsomes pooled from healthy human donors), a different radiopeptide degradation pattern was observed which was expected as a metabolism by liver enzymes involves completely different peptidases than those being present in serum. Consequently, other degradation half-lives were found for the studied radiopeptides (Figure 4, Table 1). In the line of [\(^{68}\text{Ga}\)]\(^{1}\) to [\(^{68}\text{Ga}\)]\(^{3}\), exhibiting an increasing number of artificial building blocks within the NPY sequence, the same stability trend was found as in the serum stability assay: [\(^{68}\text{Ga}\)]\(^{3}\), comprising an artificial Bip amino acid in position 32 as well as an N-terminal lauroyl modification showed the highest stability whereas the
Taking furthermore into account that the peptide shows an excellent NPY(Y1)R affinity profile, it demonstrates to be the most promising lead structure for the development of NPY(Y1) R-specific molecular imaging agents.

Conclusion

The most promising truncated NPY derivatives developed so far have been synthesized, in part modified for radiolabeling, radiolabeled with $^{68}$Ga and in the following assessed towards their stability in human serum and human microsomal stability tests. This approach of testing in both systems is not only favorable, as it covers the main physiological degradation pathways for this NPY-based peptide class, but was also shown to be important, as both methods gave in part differing and complementary results. Thus, a much more precise prediction of the in vivo stability of the tested substances is possible combining both methods compared to serum stability testing alone.

Among the studied highly promising truncated NPY analogues, [Lys(lauroyl)]$_3$Pro$_{26}$Lys(DOTA)$_{16}$Bip$_{27}$Leu$_{31}$-$^{68}$Ga$_{3}$ not only shows excellent in vitro receptor binding properties (NPY(Y1)R affinity and receptor agonism), but – in contrast to the other agents investigated – also a very high stability towards proteolytic degradation by peptidases in human serum and the human liver. It therefore represents the most promising lead compound for the further development of NPY(Y1)R-specific imaging agents.

Experimental Section

General. All commercially available chemicals were of analytical grade and were used without further purification. Resins for solid phase-based peptide syntheses, PyBOP and Fmoc-protected standard amino acids were purchased from NovaBiochem. Fmoc-$\epsilon$-Bip-OH and Fmoc-$\epsilon$-Lys(lauroyl)-OH were obtained from Iris Biotech. HBTU was purchased from Carl Roth and DOTA-tris(8Bu)ester was obtained from CheMatech. Human serum and liver microsomes were obtained from Sigma-Aldrich.

For HPLC chromatography, a Dionex UltiMate 3000 system was used together with Chromeleon Software (Version 6.80). For analytical and semipreparative chromatography, Chromolith Performance (RP-18e, 100–4.6 mm, Merck, Germany) and Chromolith Semiprep (RP-18e, 100–10 mm, Merck, Germany) columns were used, respectively. For radioanalytical use, a Dionex UltiMate 3000 system equipped with a Raytest GABI Star radioactivity detector was used together with a Chromolith Performance (RP-18e, 100–4.6 mm, Merck, Germany) column. All operations were performed using H$_2$O+$0.1\%$ TFA and MeCN+$0.1\%$ TFA as solvents at a flow rate of $4\,\text{mL/min}$. HR-ESI (high-resolution electrospray ionization) and MALDI (matrix-assisted laser desorption/ionization) spectra were obtained with Finnigan MAT95Q and Bruker Daltronics Microflex spectrometers, respectively. Gamma counting was performed by using a 2480 Wizard gamma counter system from Perkin Elmer.

General synthesis of peptides (1–5). The peptides were synthesized on solid support by standard Fmoc solid-phase peptide synthesis protocols$^{[26,27]}$, using standard commercially available
resins (Rink Amide MBHA resin, NovaSyn TG Sieber resin), HBTU as coupling agent and N_{2}N'-dicyclohexylcarbodiimide (DCC) as coupling reagent and 4 equiv. of DIEPA as base. For conjugation of DOTA-tris(6)Tuester, only 2 equiv. of acid were used, being activated with PyBOP instead of HBTU and the coupling time was prolonged to 1 h. Fmoc protecting groups were removed using 50% (v/v) piperidine in DMF within 7 min. Peptide cyclization by S→S-bond formation during the synthesis of 4 was carried out on resin applying the respective b- and L-Fmoc-Cys(Acm)-OH amino acids during peptide synthesis and 4 equiv. of Ti(TFA), in DMF (5 mL) for 45 min at ambient temperature. N-terminal acetylation of 4 was carried out using a mixture of acetic anhydride (50 equiv) and DIEPA (50 equiv) in DMF (1.5 mL) for 30 min at ambient temperature. 5 was obtained modifying a synthesis protocol for heterodisulfide-based heterodimeric peptides using DTNP. At first, the asparagine-containing peptide monomer was assembled using standard Fmoc-based SPPS methods. Analogously, the DOTA-modified peptide monomer was synthesized on solid support using Fmoc-Cys(STmp)-OH during peptide synthesis. The STmp-protecting group was removed on solid support, leaving all other protecting groups unaffected, by incubation of the resin with a solution of 2-mercaptoethanol (5%, v/v) in N-methylmorpholine in DMF (0.1 M, 5 mL) for 5 minutes. This step was repeated twice. After thorough washing of the resin with DMF, it was incubated with a solution of DTNP (5 equiv) in DMF (1 mL) for 1 h. After this time, the solution was replaced by fresh DTNP solution of the same composition and the reaction conducted for further 2 h. The resin was again washed thoroughly with DMF and incubated with a solution of asparagine-containing peptide monomer (1.25 equiv) in DMF (1 mL) overnight. The crude products were cleaved from the solid support using a mixture of TFA:TES (trisopropylsilylane):H₂O (95:2.5:2.5 v/v/v) for 60 min, suspended in diethyl ether and purified by semipreparative HPLC. The products were isolated as analytical radio-HPLC. These experiments were performed thrice for each radioligand, each experiment in triplicate.

**Determinative of the stability of [68Ga]1–[68Ga]3 and [68Ga]5 in human serum.** The peptide monomers and heterodimer were radiolabeled with 68Ga as described before and 62.5 μL of the product solutions were added to 250 μL of human serum and incubated at 37°C. At defined time-points of 5, 10, 15, 30, 60 and 90 min, aliquots of 45 μL of the mixtures of [68Ga]1–[68Ga]3 were added to 45 μL of ethanol and the precipitation of serum proteins was determined to be between 3 and 7%. After centrifugation, the supernatant was analyzed by analytical radio-HPLC. For [68Ga]5, showing a considerable adhesion to serum proteins of ~30%, the precipitation of serum proteins was omitted and the radiotracer-serum mixtures were directly analyzed by analytical radio-HPLC. These experiments were performed thrice for each 68Ga-labeled compound.

**Determination of the stability of [68Ga]1–[68Ga]3 and [68Ga]5 by microsomal stability assay.** The peptide monomers and heterodimer were radiolabeled with 68Ga as described before and the solution of the radiolabeled product was brought to pH 7.4 by addition of HEPES buffer (2 M, pH 8, ~350 μL: HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 2 μL of the product solution (~25 pmol of the respective ligand) were added to a mixture of phosphate buffer (0.05 M, pH 7.4, 800 μL) and incubated for 5 min at ambient temperature with vigorous shaking. Both phases were separated by centrifugation, and 100 μL of each phase were measured for radioactivity in a gamma-counter. From these data, the distribution coefficient logD was calculated from the following equation: logD = log(cpmp/cpme), where: cpmp = activity in the octan-1-ol phase (cpm = counts per minute) and cpme = activity in the aqueous phase. These experiments were performed thrice for each radioligand, each experiment in triplicate.

**Conclusion**

The peptides were synthesized and characterized to yield functional peptides suitable for further investigations in vivo. The radiolabeling procedure and radiochemical yields were optimized, and the labeling was shown to be efficient and reproducible. The radiolabeled compounds were characterized by MS, MALDI-TOF, and HR-ESI-MS, and the radiochemical purity was determined to be >95%. The radiolabeled peptides were shown to be suitable for in vivo applications, and further studies are needed to evaluate their potential as radiotracers for PET imaging.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: NPY(Y1)R · peptides · radiochemistry · stability · truncated NPY peptides

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