Research Article

Journal of PeptideScience

Received: 14 July 2009 Revised: 12 August 2009 Accepted: 13 August 2009 Published online in Wiley Interscience:

(www.interscience.com) DOI 10.1002/psc.1188

First selective agonist of the neuropeptide Y_1-receptor with reduced size

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Selective NPY analogues are potent tools for tumour targeting. Their Y_1-receptors are significantly over-expressed in human breast tumours, whereas normal breast tissue only expresses Y_2-receptors. The endogenous peptide consists of 36 amino acids, whereas smaller peptides are preferred because of better labelling efficiencies. As Y_1-receptor agonists enhance the tumour to background ratio compared to Y_1-receptor antagonists, we were interested in the development of Y_1-receptor selective agonists. We designed 19 peptides containing the *C*-terminus of NPY (28–36) with several modifications. By using competition receptor binding affinity assays, we identified three NPY analogues with high Y_1-receptor affinity and selectivity. Metabolic stability studies in human blood plasma of the *N*-terminally 5(6)-carboxyfluorescein (CF) labelled peptides resulted in half-lives of several hours. Furthermore, the degradation pattern revealed proteolytic degradation of the peptides by amino peptidases. The most promising peptide was further investigated in receptor activation and internalization studies. Signal transduction assays revealed clear agonistic properties, which could be confirmed by microscopy studies that showed clear Y_1-receptor internalization. For the first time, here we show the design and characterization of a small Y_1-receptor selective agonist. This agonist might be a useful novel ligand for NPY-mediated tumour diagnostics and therapeutics. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: agonist; binding affinity and selectivity; internalization; neuropeptide Y; SAR study; tumour targeting; Y_1-receptor

Introduction

The 36 amino acid peptide amide NPY belongs to the PP hormone family (also named neuropeptide Y family) together with PP and PYY. It is one of the most abundant peptides in the brain and is expressed in the peripheral and central nervous system [1–3]. In addition, numerous physiological functions are mediated by NPY, such as the induction of food intake, the vasoconstriction, the inhibition of anxiety, the increase in memory retention and the inhibition of alcohol consumption and resistance [4–11]. These functions are transmitted by four Y-receptors (Y_1-, Y_2-, Y_4- and Y_5-receptor) which all belong to the class A of GPCR [12–14]. NPY activates Y_1- and Y_2-receptors with nanomolar affinity followed by internalization, whereas the activation of Y_5-receptors does not lead to internalization [15].

Furthermore, recently a novel function has been identified for Y-receptors. The expression of Y-receptors has been found in several neuroblastoma as well as in gastrointestinal stromal tumours, renal cell carcinoma and breast carcinoma [16–19]. Moreover, a change of Y-receptor expression has been identified during neoplasm from normal breast tissue to breast cancer tissue [16]. Normal breast tissue expresses Y_2-receptors, whereas Y_1-receptors are strongly over-expressed in breast tumours. Accordingly, receptor subtype selective NPY analogues can be used as a next generation of agents in tumour diagnosis and therapy.

At present different *full-length* NPY agonists are available showing high Y_1-receptor selectivity compared to other Y-receptors, e.g. [Leu^{31}, Pro^{34}]NPY and [Phe^{7}, Pro^{34}]NPY [20,21]. Previous studies in our work group confirmed the application of selective Y_1-agonists in tumour labelling by synthesizing [Phe^{7}, Pro^{34}]NPY with the BFC DOTA [22]. The data showed a significant labelling dependence on the position of the BFC. *In vivo* biodistribution studies demonstrated a clear uptake of the ^{111}In-DOTA labelled NPY analogue in the tumour in human breast adenocarcinoma (MCF-7) tumour-bearing mice. Accordingly, we could nicely show the proof of principle.

Unfortunately, large peptides like the 36 amino acid NPY analogue [Phe^{7}, Pro^{34}]NPY are expensive to synthesize. Furthermore, the labelling efficiency usually is much better for smaller peptides. Therefore, smaller ligands are required to efficiently label the tumour. Up to now, many small peptidic and non-peptidic Y_1-receptor ligands have been developed and characterized, such as the peptide ligands GR231118 (also known as 1229U91 and GW1229), T-241, T-190, BW1911U90, [$^{32,34}\beta$ACC] NPY (25–36) and the non-peptide antagonists BIBP3226, BIBO3304 and LY357 897 (Figure 1) [23–29]. BIBP3226 was the first non-peptidic Y_1-receptor ligand, whereas BIBO3304 shows a tenfold higher Y_1-receptor affinity compared to BIBP3226 [29]. Peptidic ligands, e.g. GR231118 mimic the *C*-terminal part of NPY which is essential for receptor binding [30–33] or contain a large *C*-terminal segment like [^{31}R,$^{32,34}\beta$ACC] NPY (25–36) or [$^{32,34}\beta$ACC] NPY (25–36)

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Abbreviations used: *βACC, β-aminocyclopropane carboxylic acid; BFC, bifunctional chelator; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; GPCR, G-protein coupled receptor; h, human; p, porcine; PP, pancreatic polypeptide; PYY, peptide YY.*

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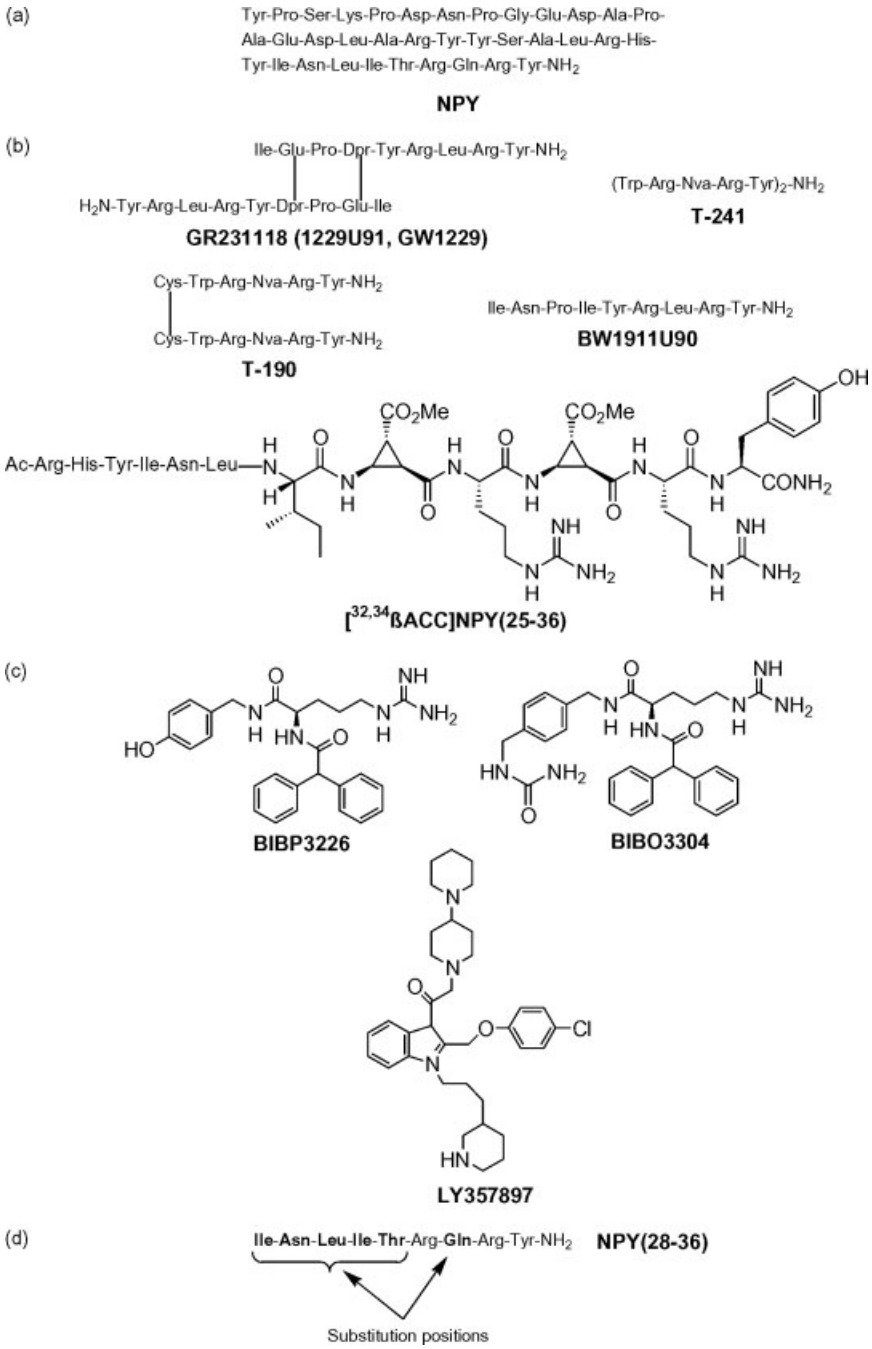

Figure 1. (a) Sequence of porcine neuropeptide Y (pNPY). (b) Structures of the peptidic Y_1-receptor selective antagonists GR231118 (1229U91, GW1229), T-241, T-190, BW1911U90 and [$^{32,34}\beta$ACC] NPY (25–36). (c) Structures of the non-peptidic Y_1-receptor selective antagonists BIBP3226, BIBO3304 and LY357897. (d) Concept for the development of peptidic Y_1-receptor selective agonists based on the *C*-terminal segment of NPY.

[26]. However, these reduced size ligands show high affinity but are not able to activate the receptor, and accordingly are Y_1-receptor antagonists. Moreover, the peptidic Y_1-receptor antagonist GR231118 also activates Y_4-receptors and accordingly lacks selectivity [34–36]. As receptor internalization is only induced by agonists [15,37,38] and this internalization is an important prerequisite to improve the tumour to background ratio, agonists are required for this type of experiments. Previously, centrally truncated NPY analogues, such as Des-AA$^{11-18}$[Cys^{7,21}, D-Lys^{9} (Ac), D-His^{26}, Pro^{34}]NPY, have been described with high Y_1-receptor affinities and agonistic properties [39]. But these peptides are still too large for the successful application as tumour marker. Therefore, smaller Y_1-receptor selective agonists are required as a next generation.

In the present study, we designed peptides based on the knowledge of diverse SAR studies of NPY. These studies showed that proline30 improves Y_1-receptor binding and leucine34 enhances the helicity of the peptide, respectively [20,24]. Furthermore, we know from *full-length* NPY that positions 31 and 32 are relevant for agonistic properties [21]. Accordingly, we synthesized a set of 19 NPY analogues containing nine *C*-terminal amino acids of NPY (Figure 1(d)). To obtain Y_1-receptor

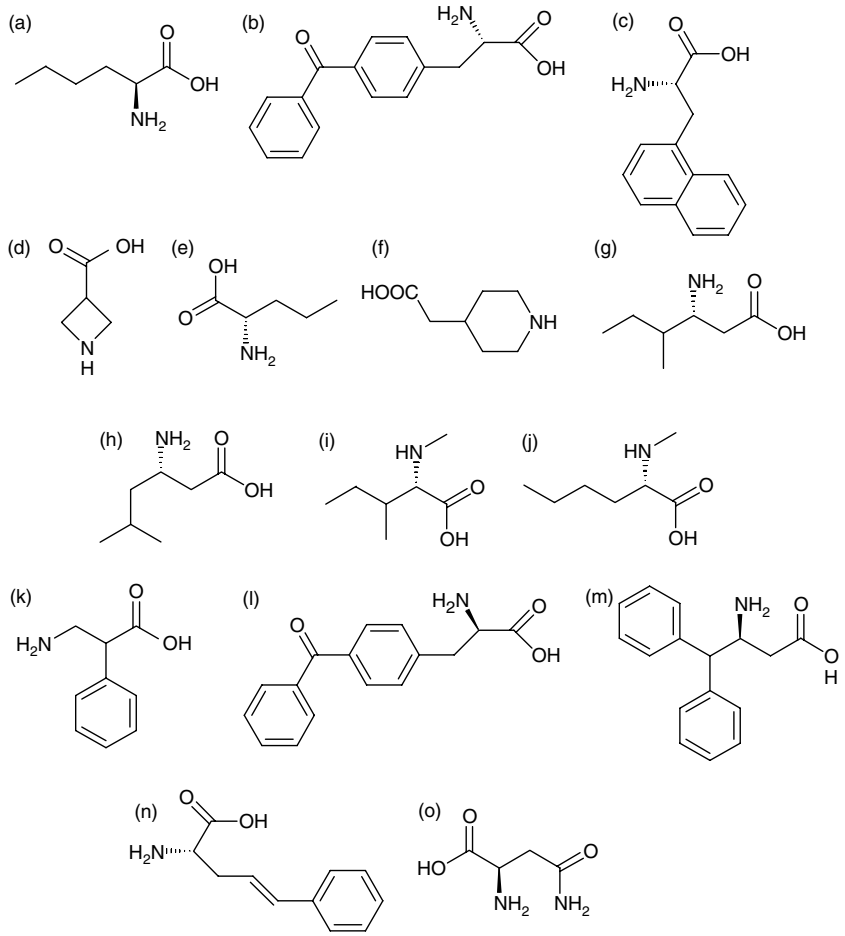

Figure 2. Chemical structures of the non-proteinogenic amino acids used to generate novel NPY analogues. (a) norleucine (Nle). (b) L-4-benzoylphenylalanine (Bpa). (c) 3-(1-naphthyl)-L-alanine (Nal). (d) azetidin-3-carbon acid (Aze). (e) norvaline (Nva). (f) piperidin-4-yl acetic acid (Pip). (g) β-homo-isoleucine (βhomoIle). (h) β-homo-leucine (βhomoLeu). (i) *N*-methyl-isoleucine (NMeIle). (j) *N*-methyl-norleucine (NMeNle). (k) 2-phenyl-β-alanine (pβAla). (l) D-4-benzoylphenylalanine (DBpa). (m) γ,γ-diphenyl-D-β-homo-alanine (DPβhomoAla). (n) 3-styrylalanine (βstyrylAla). (o) D-asparagine (DAsn).

selective agonists we modified especially the positions 31 and 32 by using 15 non-proteinogenic amino acids (Figure 2). A loss of affinity has been observed for 16 of the 19 peptides, whereas three NPY analogues (**1a, 2a** and **3a)** selectively bind to Y_1-receptors with nanomolar affinities. *In vitro* metabolic stability studies revealed half-lives of several hours as well as a proteolytic degradation at arginine33 and arginine35. Interestingly, the NPY analogue **1a** showed full agonistic activity in Y_1-receptor activation assays. This result could be confirmed by microscopy studies which exhibited a clear peptide-mediated Y_1-receptor internalization.

Materials and Methods

Materials

The N^{α}-Fmoc-protected proteinogenic amino acids as well as the N^{α}-Fmoc-protected non-proteinogenic amino acids were obtained from Alexis (Läufelfingen, Switzerland), Novabiochem (Läufelfingen, Switzerland) and Fluka (Buchs, Switzerland). The 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink amide) resin was obtained from Novabiochem. DMF, DCM, methanol (MeOH) and diethylether (Et_2O) were purchased from Scharlau (La Jota, Barcelona, Spain). CF, HOBt, thioanisole, *p*-thiocresol, piperidine, diisopropylethylamine, hydrazine monohydrate, *tert*.-butanol and HATU were purchased from Fluka. *N-N′*-diisopropylcarbodiimide (DIC) and DIPEA were purchased from Aldrich (Buchs, Switzerland). Acetonitrile (ACN) was obtained from Romil (Cambridge, England). TFA was obtained from Fluka. Dulbecco's MEM : NutMix F12 medium (50 : 50, *v*/*v*), Dulbecco's PBS, sodium pyruvate, fetal calf serum (FCS) and EDTA were purchased from Gibco (Life Technologies, Basel, Switzerland). Hank's balanced salt solution (HBSS), penicillin, streptomycin and L-glutamine were obtained from PAA laboratories (Pasching, Austria). Metafectene™ was obtained from Biontex Laboratories GmbH (Martinsried, Planegg, Germany). Hygromycin B was purchased from Merck (Darmstadt, Germany). Fluoromount G was from Carl Roth GmbH & Co. KG (Wiesbaden, Germany). Hoechst 3342, lithium chloride (LiCl) and paraformaldehyde (PFA) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Bacitracin and BSA were from Sigma (Buchs, Switzerland), while Pefabloc was from Fluka. Chimeric G proteins were kindly provided by E. Kostensis. [^{3}H]-hPP with a specific activity of 85 Ci/mmol was obtained by selective labelling as described [40]. Lys^4(N^{ε}-[propionyl-^{3}H])-pNPY and *myo*-[2-^{3}H]-inositol were obtained from GE Healthcare Europe GmbH (Braunschweig, Germany).

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Peptide Synthesis

Peptides were synthesized with an automated multiple peptide synthesis robot system (Syro, MultiSynTech, Bochum, Germany) by Fmoc/*t*-Bu strategy using the Rink amide resin (30 mg with a resin loading of 0.6 mmol/g). For amino acid coupling a tenfold excess of the N^{α}-Fmoc-protected amino acid was activated *in situ* with 10 equiv DIC and HOBt (0.5 M) in DMF. Coupling was achieved within 40 min and repeated once. The Fmoc-group was cleaved with piperidine (40%) in DMF for 3 min and with piperidine (20%) in DMF for 10 min. After peptide synthesis, cleavage from the resin and of the side chain protecting groups was performed with TFA (90%) and scavenger (thioanisole : thiocresol 1 : 1) (10%) for 3 h at room temperature. Peptides were purified by a preparative RP-HPLC system (Shimadzu RP18-column, 12.5 × 250 mm, 5 µm/300 Å, Columbia, MD, USA). The pure peptides were characterized by analytical RP-HPLC (Vydac RP18-column, 4.6 × 250 mm, 5 µm/300 Å, Merck Hitachi, Darmstadt, Germany) by using a linear gradient of 0.1% TFA in water (A) and 0.08% TFA in ACN (B) from 10% to 60% over 30 min at a flow rate of 0.6 ml/min. For peptides **1a, 2a** and **3a** a second gradient from 20% to 50% over 30 min at a flow rate of 0.6 ml/min was used. The identity was confirmed by Maldi-ToF mass spectrometry (Ultraflex III MALDI-TOF/TOF, Bruker Daltonics, Billeria, MA, USA).

The non-proteinogenic amino acids were introduced into the peptide by a manual coupling procedure. A twofold excess of the N^{α}-Fmoc-protected non-proteinogenic amino acid was activated *in situ* with 2 equiv HATU and 1 equiv DIPEA and the resin-bound peptide in DMF (500 µl) for 1 h at room temperature in a double coupling step. Then the resin was washed three times with DMF, DCM, MeOH and Et_2O and dried.

For metabolic stability studies peptides **1a, 2a, 3a** and **20a** were *N*-terminally labelled with CF to obtain peptides **1b, 2b, 3b** and **20b**. A tenfold excess of the CF was incubated with 10 equiv HOBt, 10 equiv DIC and the resin-bound peptide in DMF (500 µl) for 2 h. Then, the resin was washed three times with DMF, DCM, MeOH and Et_2O and dried. Cleavage of the peptide from the resin and identification was performed as described above.

Analytical data of the peptides are summarized in Table 1.

Cell Culture

All cells were grown in a humidified atmosphere at 37 °C and 5% CO_2 to confluence before use. MCF-7 (ATCC), SMS-KAN [41,42] and HEC-1b-hY_5 [43] (kindly provided by Prof. A. Buschauer, University of Regensburg, Regensburg, Germany) cells were cultured in DMEM : nutrient mix Ham's F12 (1 : 1) containing 10% FCS (MCF-7) or 15% FCS (SMS-KAN and HEC-1b-hY_5) and 2 mM L-glutamine. COS-7 cells (ATCC) were cultured in DMEM containing 10% FCS, 100 units/ml penicillin and 100 µl/ml streptomycin. HEK293 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany) stably transfected with the human Y_1-receptor and labelled with EYFP as described elsewhere [15] were cultured in DMEM : Ham's F12 (1 : 1) without L-glutamine containing 15% FCS and 100 µg/ml hygromycin B.

Table 1. Analytical and biological data of the synthesized peptides

No.	Peptide	$MW_{calc.}$	$MW_{expt.}$	RT[a]	RT[b]	IC_{50} [nM] NPY Y_1
1a	[Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1294.0	1295.0	19.3	16.3	29.7 ± 6.8
1b	CF-[Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1652.0	1653.0	24.8	n.d.	n.d.
2a	[Pro^{30}, Nal^{32}, Leu^{34}]NPY(28–36)	1240.0	1241.0	18.3	15.8	94.3 ± 1.6
2b	CF-[Pro^{30}, Nal^{32}, Leu^{34}]NPY(28–36)	1598.0	1599.0	23.6	n.d.	n.d.
3a	[Pro^{30}, Nle^{31}, Nal^{32}, Leu^{34}]NPY(28–36)	1240.0	1241.0	19.6	15.8	70.1 ± 3.9
3b	CF-[Pro^{30}, Nle^{31}, Nal^{32}, Leu^{34}]NPY(28–36)	1598.0	1598.0	24.4	n.d.	n.d.
4	[Aze^{30}, Tyr^{32}, Leu^{34}]NPY(28–36)	1191.0	1192.0	18.7	n.d.	>1000
5	[Aze^{30}, Tyr^{32}, Nva^{34}]NPY(28–36)	1175.0	1176.0	19.0	n.d	>1000
6	[Aze$^{29-30}$, Tyr^{32}, Leu^{34}]NPY(28–36)	1300.0	1301.0	19.1	n.d	>1000
7	[Aze$^{29-30}$, Tyr^{32}, Nva^{34}]NPY(28–36)	1062.0	1063.0	18.3	n.d.	>1000
8	[Pip^{30}, Tyr^{32}, Leu^{34}]NPY(28–36)	1234.0	1235.0	18.2	n.d.	>1000
9	[Pip$^{29-30}$, Tyr^{32}, Leu^{34}]NPY(28–36)	1341.0	1342.0	19.7	n.d.	>1000
10	[Pro^{30}, βhomoIle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1308.0	1309.0	19.2	n.d.	589.9 ± 53.1
11	[Pro^{30}, βhomoLeu^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1308.0	1309.0	19.2	n.d.	>1000
12	[Pro^{30}, NMeIle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1308.0	1309.0	19.4	n.d.	>1000
13	[Pro^{30}, NMeNle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1308.0	1309.0	19.4	n.d.	705.6 ± 38.8
14	[Pro^{30}, pβAla^{32}, Leu^{34}]NPY(28–36)	1190.0	1191.0	18.9	n.d.	>1000
15	[Pro^{30}, DBpa^{32}, Leu^{34}]NPY(28–36)	1294.0	1295.0	18.8	n.d.	>1000
16	[Pro^{30}, DPβhomoAla^{32}, Leu^{34}]NPY(28–36)	1280.0	1281.0	19.5	n.d.	>1000
17	[Pro^{30}, βstyrylAla^{32}, Leu^{34}]NPY(28–36)	1216.0	1217.0	19.3	n.d.	>1000
18	[NMeIle^{28}, Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1308.0	1309.0	19.8	n.d.	521.4 ± 47.5
19	[DAsn^{29}, Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	1294.0	1295.0	19.3	n.d.	>1000
20a	pNPY	4254.0	4255.0	22.1	n.d.	2.0 ± 0.4
20b	CF-pNPY	4612.0	4613.0	18.1	n.d.	n.d.

n.d., not determined.
[a] Retention time from 10 to 60% ACN (0.08% TFA) in water (0.1% TFA) over 30 min.
[b] Retention time from 20 to 50% ACN (0.08% TFA) in water (0.1% TFA) over 30 min.

Receptor Binding Affinity Assay

Receptor binding assays were performed in a competition assay using 1 nM $Lys^4(N^{\varepsilon}$-[propionyl-^{3}H])-pNPY (GE Healthcare Europe GmbH) and increasing concentrations of the respective peptide ($10^{-5}-10^{-10}$ M) as described previously [22]. For binding assays at Y_4-receptors COS-7 cells were seeded into 25 cm^2 flasks. At 60–70% confluency COS-7 cells were transiently transfected with 4 µg vector DNA and 15 µl Metafectene™. Twenty-four hours after transfection receptor binding affinity assays were performed using 1 nM [^{3}H]-propionylated hPP ([^{3}H]-hPP) and increasing concentrations of the respective peptide ($10^{-5}-10^{-10}$ M). [^{3}H]-hPP with a specific activity of 85 Ci/mmol was achieved by selective labelling as described recently [40]. The obtained data were analysed with GraphPad Prism 3.0 program (GraphPad Software, San Diego, USA). Experiments were performed in triplicate and $IC_{50} \pm$ SEM values were calculated.

In Vitro Metabolic Stability

For metabolic stability studies the CF labelled peptides **1b, 2b, 3b** and **20b** (10 µM) were incubated in human blood plasma (0.1 ml) obtained from healthy donors for 0, 0.5, 1, 1.5, 2, 4, 6, 12 h as well as for 24 and 48 h (CF-NPY). Samples were precipitated with ACN : ethanol (1 : 1) (40 µl) followed by centrifugation at 8000 rpm and cooling at 4 °C for 6 h. The suspension was centrifuged for 15 min at 14 000 rpm and the supernatant was filtered through 45 µm and 22 µm filters. Probes were analysed by RP-HPLC (Vydac RP18-column, 4.6 × 250 mm, 5 µm/300 Å, Merck Hitachi) and monitored by fluorescence detection. A linear gradient with 0.1% TFA in water (A) and 0.08% TFA in ACN (B) from 10 to 60% B in A over 40 min at a flow rate of 0.6 ml/min was used. Experiments were performed in triplicate and $t_{1/2} \pm$ SEM values were calculated. The fluorescently labelled metabolites were collected from each HPLC chromatogram and identified by MALDI-ToF mass spectrometry (Ultraflex III MALDI-TOF/TOF, Bruker Daltonics) as possible cleavage sites from the parent peptide.

Signal Transduction Assay

For signal transduction assays COS-7 cells were seeded into 24-well plates (1.0×10^5 cells/well) and transiently transfected with plasmid hY_1-receptor DNA (0.2 µg) and plasmid DNA coding for the chimeric $G\alpha_{qi4}$-protein (0.05 µg) (kindly provided by E. Kostensis) by using Metafectene™. Incubation with myo-[2-^{3}H]-inositol (2 µCi/ml) (GE Healthcare Europe GmbH) was performed 1 day after transfection for 16 h. Cells were washed once and stimulated with increasing concentrations of each peptide for 1 h at 37 °C in DMEM/LiCl (10 mM). The receptor stimulation was stopped by aspiration of the medium followed by cell lysis with NaOH (0.1 M, 300 µl). After addition of formic acid (0.2 M, 100 µl) and sample dilution intracellular IP levels were determined by anion-exchange chromatography. Obtained data were analysed with GraphPad Prism 3.0 program (GraphPad Software). Experiments were performed in triplicate and $EC_{50} \pm$ SEM values as well as $E_{max} \pm$ SEM values were calculated.

Microscopy Study

For microscopy studies HEK293-hY_1R-EYFP [15] cells were transferred into 24-well plates (3.0×10^5 cells/well) containing sterilized glass cover slips. After 24 h cells were starved for 60 min and stimulated with each peptide (1 µM) for 1 h at 37 °C. The stimulation was terminated by washing the cells with HBSS and cells were fixed with 4% PFA for 30 min at room temperature. The nuclei were visualized with Hoechst 33 342 (1 µg/ml) for 10 min at room temperature. Glass cover slips were mounted on microscope slides with Fluoromount G. Fluorescence images were obtained as previously described [15].

Results

Design of Novel Peptides

We designed a set of 19 nonapeptides with multiple amino acid exchanges derived from the *C*-terminal part of NPY (28–36). The amidated *C*-terminus as well as the arginine residues 33 and 35 were kept constant, because they have been shown to be essential for receptor binding [32,44]. The positions 28–32 and the position 34 were substituted by different proteinogenic and non-proteinogenic amino acids. Position 34 was changed in all peptides from glutamine of the original sequence to the unpolar and aliphatic amino acids leucine or norvaline. Positions 31 and 32 were substituted systematically by different non-proteinogenic amino acids as shown in Figure 2. Replacements of these positions have been shown before to be able to modify activity and large amino acids have been shown to be favourable [21,45]. Peptides were synthesized by SPPS by the Fmoc/*t*-Bu strategy. The introduction of the non-proteinogenic amino acids was achieved manually to the resin-bound peptides. Then, the peptides were purified by preparative RP-HPLC to obtain peptides with >98% purity. The characterization was performed by analytical RP-HPLC. For the most promising peptides **1a, 2a** and **3a** a second RP-HPLC gradient was used to confirm homogeneity. The identity was confirmed by Maldi-ToF-MS. The analytical data of the peptides **1a, 2a** and **3a** are shown in Figure 3.

Receptor Binding and Selectivity

The competition receptor binding affinity assays of the peptides were performed at cell lines which selectively express specific Y-receptors. MCF-7 (human breast adenocarcinoma) cells express Y_1-, SMS-KAN (human neuroblastoma) cells [41,42] express Y_2-, HEC-1b-hY_5 (human endometrial carcinoma) cells [43] express Y_5- and transfected COS-7 (African green monkey, kidney) cells express Y_4-receptors. In a first approach we analysed the Y_1-receptor affinities of the 19 synthesized peptides by the determination of their IC_{50}-values. 16 of the 19 NPY analogues were found to show low affinity or were unable to displace ^{3}H-NPY from Y_1-receptors with IC_{50}-values >1000 nM. Peptides **10**, **13** and **18** exhibited some Y_1-receptor affinities (IC_{50}-values of 589.9 ± 53.1, 705.6 ± 38.8 and 521.4 ± 47.5 nM). But three NPY analogues, peptides **1a, 2a** and **3a**, showed significant Y_1-receptor affinities. For peptide **1a** only a 15-fold reduced binding affinity was observed compared to *full-length* NPY, although this peptide contains only 9 compared to 36 amino acids (**20a**) (IC_{50}-values of 29.7 ± 6.8 vs 2.0 ± 0.4 nM). Similar results with minor reduced binding affinities at Y_1-receptors were obtained for peptides **2a** and **3a** (IC_{50}-values of 94.3 ± 1.6 and 70.1 ± 3.9 nM). Table 1 summarizes the IC_{50}-values of the 19 NPY analogues at Y_1-receptors.

Next, we investigated the receptor selectivity of the Y_1-receptor active peptides **1a, 2a** and **3a**. For all three peptides we identified

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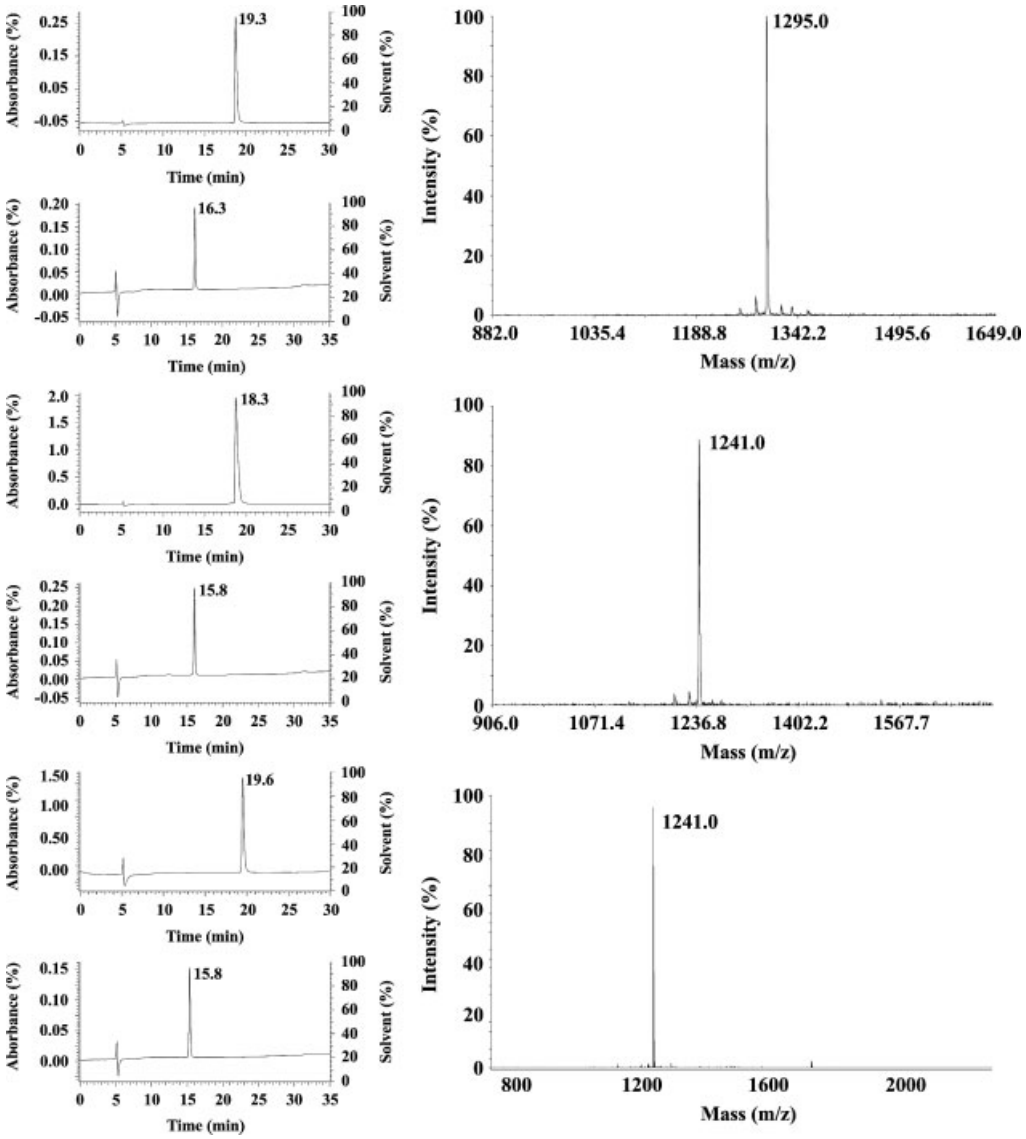

Figure 3. Analytical data of peptides **1a** (a,b), **2a** (c,d) and **3a** (e,f). Peptide **1a**: (a) Analytical RP-HPLC chromatogram using a gradient from 10 to 60% ACN (0.08% TFA) in water (0.1% TFA) over 30 min (t_R = 19.3 min) (up) and analytical RP-HPLC chromatogram using a gradient from 20 to 50% ACN (0.08% TFA) in water (0.1% TFA) over 30 min (t_R = 16.3 min) (down). (b) Maldi-ToF mass spectrum with an expt. mass ($M + H^+$) of 1295.0 Da (calc. mass of 1294.0 Da). Peptide **2a**: (c) Analytical RP-HPLC chromatogram using a gradient from 10 to 60% ACN (0.08% TFA) in water (0.1% TFA) over 30 min (t_R = 18.3 min) (up) and analytical RP-HPLC chromatogram using a gradient from 20 to 50% ACN (0.08% TFA) in water (0.1% TFA) over 30 min (t_R = 15.8 min) (down). (d) Maldi-ToF mass spectrum with an expt. mass ($M + H^+$) of 1241.0 Da (calc. mass of 1240.0 Da). Peptide **3a**: (e) Analytical RP-HPLC chromatogram using a gradient from 10 to 60% ACN (0.08% TFA) in water (0.1% TFA) over 30 min (t_R = 19.6 min) (up) and analytical RP-HPLC chromatogram using a gradient from 20 to 50% ACN (0.08% TFA) in water (0.1% TFA) over 30 min (t_R = 15.8 min) (down). (f) Maldi-ToF mass spectrum with an expt. mass ($M + H^+$) of 1241.0 Da (calc. mass of 1240.0 Da).

significantly decreased binding affinities at Y_2-, Y_4- and Y_5-receptors compared to the Y_1-receptor, however the peptides behaved slightly different. Peptide **1a** showed the most favourable Y_1-receptor selectivity with IC_{50}-values >1000 nM for the Y_2-, Y_4- and Y_5-receptor. For peptides **2a** and **3a** a significant loss in binding affinities was found at Y_2- and Y_5-receptors, but they showed a weak binding affinity at Y_4-receptors compared to the native ligand PP (IC_{50}-values of 140.8 ± 15.2 and 435.1 ± 8.7 vs 3.4 ± 0.1 nM). Receptor selectivity data of the peptides **1a, 2a** and **3a** are shown in Table 2.

In vitro Metabolic Stability

To investigate the *in vitro* metabolic stability, peptides **1a, 2a, 3a** as well as NPY (**20a**) were *N*-terminally labelled with CF to obtain peptides **1b, 2b, 3b** and **20b**. Then, the CF labelled peptides were incubated in human blood plasma and after different time intervals the metabolic reaction was stopped by addition of acid. The degradation level was determined by fluorescence detection of the CF group after analytical RP-HPLC. Peptides **1b, 2b** and **3b** showed four- to fivefold reduced half-lives compared to CF-NPY (**20b**) ($t_{1/2}$ of 4.9 ± 0.3, 4.8 ± 1.6 and 5.6 ± 3.5 vs 24.7 ± 1.4 h). Table 3 shows the half-lives of the CF labelled peptides as well as of CF-NPY (**20b**). Furthermore, we identified the main cleavage products of the peptides **1b, 2b** and **3b** by separation of the detected peaks and subsequent characterization by Maldi-ToF mass spectrometry. Two major cleavage products could be detected for all three peptides: *N*-terminal cleavage at position arginine33 and arginine35. As an example, Figure 4 shows

Table 2. Binding affinity and selectivity of the most potent peptides

No.	Peptide	IC_{50} [nM] NPY Y_1	NPY Y_2	NPY Y_4	NPY Y_5
1a	[Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	29.7 ± 6.8	>1000	>1000	>1000
2a	[Pro^{30}, Nal^{32}, Leu^{34}]NPY(28–36)	94.3 ± 1.6	>1000	140.8 ± 15.2	>1000
3a	[Pro^{30}, Nle^{31}, Nal^{32}, Leu^{34}]NPY(28–36)	70.1 ± 3.9	>1000	435.1 ± 8.7	>700
20a	pNPY/hPP	2.0 ± 0.4	4.8 ± 0.3	3.4 ± 0.1 (PP)	9.9 ± 0.2

Table 3. Half-lives in human blood plasma of the most potent peptides

No.	Peptide	$t_{1/2}$ (h)
1b	CF-[Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–36)	4.9 ± 0.3
2b	CF-[Pro^{30}, Nal^{32}, Leu^{34}]NPY(28–36)	4.8 ± 1.6
3b	CF-[Pro^{30}, Nle^{31}, Nal^{32}, Leu^{34}]NPY(28–36)	5.6 ± 3.5
20b	CF-pNPY	24.7 ± 1.4

the degradation pattern with the two main cleavage products, CF-[Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28–34) and CF-[Pro^{30}, Nle^{31}, Bpa^{32}]NPY(28–32), of peptide **1b** after incubation with human blood for 12 h.

Receptor Activation and Internalization

As peptide **1a** was identified to be the most promising peptide after receptor binding and selectivity studies as well as *in vitro* metabolic stability studies we used this peptide for further investigations of Y_1-receptor activation and internalization. By the substitution of the four or five *C*-terminal amino acids of $G\alpha_q$ with the corresponding $G\alpha_i$ to obtain $G\alpha_{qi4}$ it is possible to stimulate the phospholipase C_β pathway onto G_i-coupled receptors [46,47]. Peptide **1a** was tested for signal transduction properties on COS-7 cells transiently transfected with plasmid hY_1-receptor DNA and plasmid DNA that codes for the chimeric $G\alpha_{qi4}$-protein. As shown in Figure 5(b), the results confirm the binding potency at Y_1-receptors determined in competition receptor binding affinity assays (EC_{50}-value of 65.7 ± 8.9 nM; E_{max}-value of 0.9 ± 0.1 for **1a** as

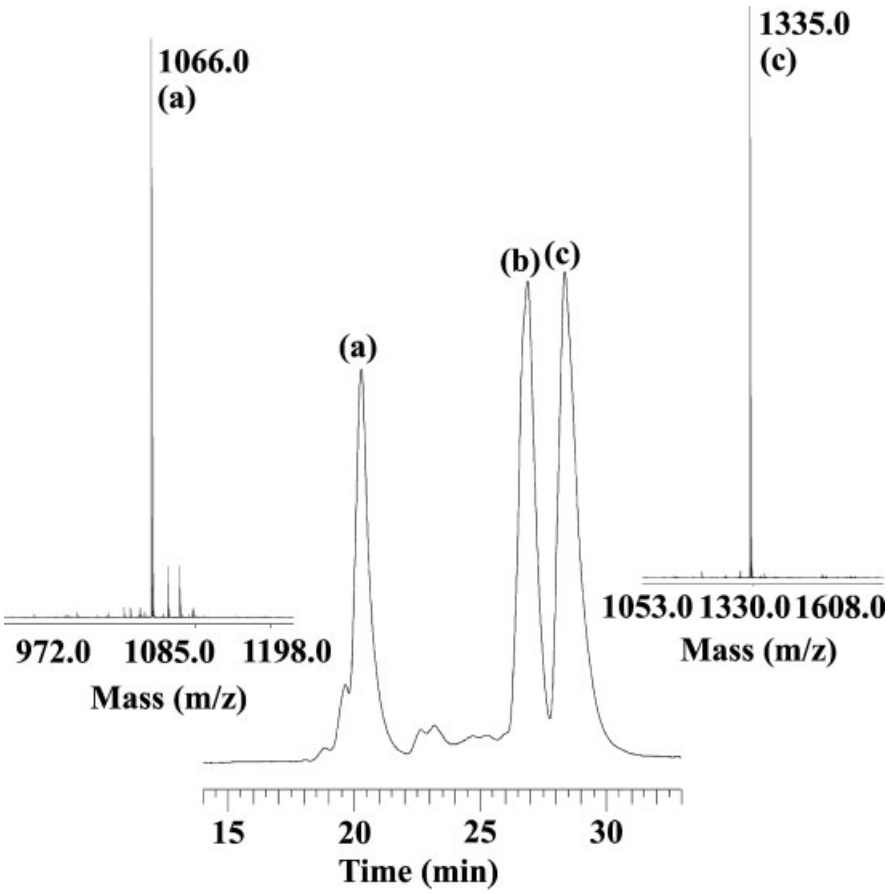

Figure 4. *In vitro* metabolic stability in human blood plasma – degradation pattern of peptide **1b**. Detection after 12 h incubation at 37 °C. Degradation analysed by analytical HPLC and identification of the cleavage products by Maldi-ToF mass spectrometry. (a) Cleavage product CF-[Pro^{30}, Nle^{31}, Bpa^{32}]NPY(28–32) (b) Intact peptide **1b**. (c) Cleavage product CF-[Pro^{30}, Nle^{31}, Bpa^{32}]NPY(28–32).

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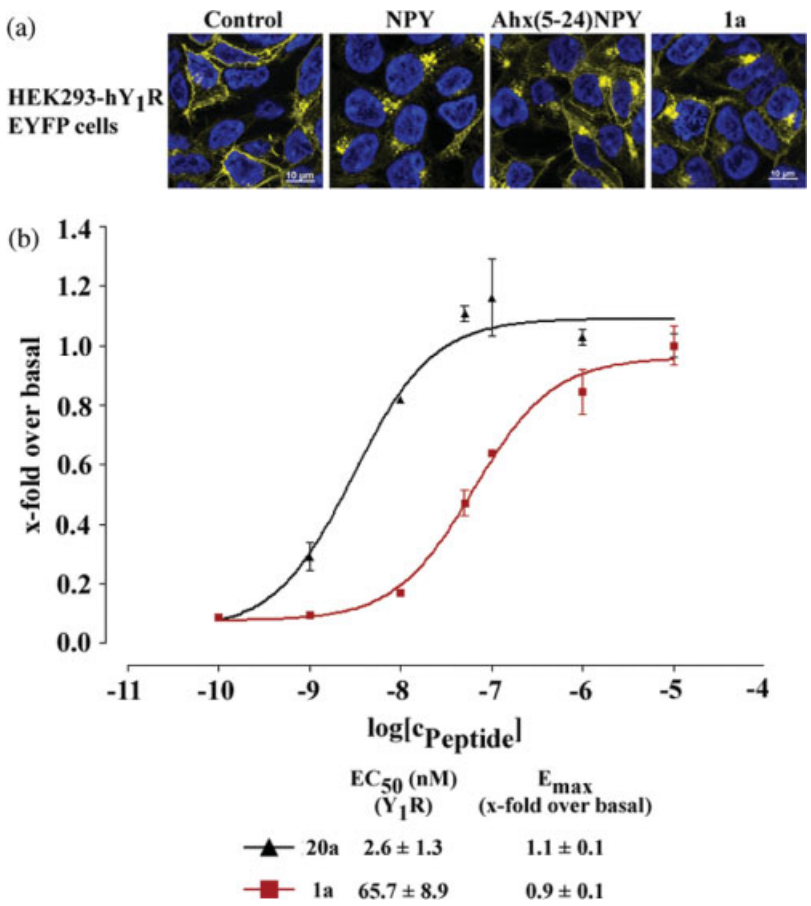

Figure 5. Peptide **1a**-mediated Y_1-receptor internalization. (a) HEK293 cells stably transfected with hY_1R-EYFP (receptors marked in yellow) were incubated with 1 μM of NPY (**20a**), Ahx(5–24)NPY or peptide **1a** for 60 min. (b) COS-7 cells transiently co-expressing hY_1 and $G\alpha_{qi4}$ and incubated with increasing concentrations of NPY (**20a**) as well as peptide **1a**. Data are shown as x-fold over basal IP levels. EC_{50}-values and E_{max}-values were obtained from the concentration response curves of the ligands.

well as EC_{50}-value of 2.6 ± 1.3 nM; E_{max}-value of 1.1 ± 0.1 for **20a**). Furthermore, the sigmoidal response curve of peptide **1a** showed a strong agonistic activity, which almost reaches full agonism.

In order to confirm this result we used microscopic studies to investigate the peptide **1a-**mediated Y_1-receptor internalization. Therefore, stably transfected HEK293-hY_1R-EYFP cells [15] were incubated with peptide **1a.** As a positive control NPY (**20a**) was used. For negative controls only media and the Y_2-receptor selective NPY analogue Ahx(5–24)NPY [48–50] were applied. By using NPY (**20a**) a complete peptide-mediated Y_1-receptor internalization could be observed, whereas the two negative controls only showed membrane localization of the EYFP labelled Y_1-receptor. For peptide **1a**, however, we clearly could show Y_1-receptor internalization with some weak membrane localized Y_1-receptors (Figure 5(a)). This result is essential for a possible application of this peptide in tumour targeting.

Discussion

The *C*-terminal part of NPY is crucial and directly involved in Y_1-receptor binding [30–33,51]. Previously, different peptidic Y_1-receptor antagonists which mimic the *C*-terminus of NPY have been developed [25,26,28]. The potent Y_1-receptor antagonist GR231118, a symmetrical dimeric peptide, has been synthesized and its role in various physiological effects has been determined [24,52–54]. Unfortunately, GR231118 is not only a Y_1-receptor antagonist, but also a Y_4-receptor agonist [34–36]. But this small peptidic ligand turned out to be a useful tool for the development of more potent Y_1-receptor selective peptides with agonistic properties. For the successful application as tumour marker Y_1-receptor selective agonists are required. In previous studies receptor internalization has been only revealed for agonists, whereas no receptor internalization has been observed for antagonists [15]. In the field of tumour markers, agonism results in higher tumour to background ratios. Unfortunately, all known small Y_1-receptor selective peptides are non-peptides or antagonists. Furthermore, the application is not only limited to tumour diagnosis, because Y_1-receptor selective agonists can be used as cytotoxic (antineoplastic) drug delivery agents. The reduction of the cytotoxic toxicity resulted then by the selective internalization of the peptide.

The knowledge of GR231118 provided as starting point of our SAR study to obtain small Y_1-receptor selective agonists. We developed a set of 19 truncated NPY analogues. All peptides contain the *C*-terminal amino acids 28–36 of NPY with several amino acid exchanges, including 15 different non-proteinogenic amino acids. It has been shown that the loss of the *C*-terminal $CONH_2$ group leads to a not acceptable reduction of Y_1- and Y_2-receptor affinity [44]. Furthermore, an L-alanine scan of NPY revealed that especially the arginine33 and the arginine35 are

essential for receptor affinity and activity [32]. Therefore, we did not modify these three positions. As a D-amino acid scan of NPY showed similar changes to the L-alanine scan, we suggested that not only the side chain of the amino acid itself but also their orientation is important [55]. The substitution of leucine30 to proline30 of NPY has been shown to enhance the Y_1-receptor binding, whereas the substitution of glutamine34 to leucine34 revealed an improved helicity of the peptide [20,24]. The positions 31 and 32 of NPY were found to be able to modify the agonistic property of the peptide [21]. Consequently, our study focused on the modification of these positions to determine small Y_1-receptor selective agonists. The position 31 was substituted by unpolar/aliphatic non-proteinogenic amino acids. It has been hypothesized that the substitution of threonine32 to tyrosine32 mimics tyrosine1 in *full-length* NPY and improves the peptide-receptor interaction [45]. Accordingly, the position 32 was modified by aromatic proteinogenic and non-proteinogenic amino acids.

Sixteen of the nineteen synthesized peptides showed a loss in Y_1-receptor affinity. However, three NPY analogues, peptides **1a, 2a** and **3a**, showed strong nanomolar binding affinities at Y_1-receptors. Peptide **1a** exhibited the best Y_1-receptor affinity with an IC_{50} value of 29.7 $\pm$ 6.8 nM which is only 15-fold reduced compared to NPY. Peptides **2a** and **3a** are characterized by a 35–48-fold loss in Y_1-receptor affinity compared to NPY as shown by the determined IC_{50} values of 94.3 $\pm$ 1.6 nM for peptide **2a** and 70.1 $\pm$ 3.9 nM for peptide **3a**. Interestingly, the difference of peptide **2a** to peptide **3a** is only the exchange of isoleucine31 containing a β-branched side chain to the unbranched amino acid norleucine31. Our results are in agreement with the hypothesis of previous studies that the 3D arrangement and orientation of the amino acid and the side chain are important for high binding affinity [56]. Furthermore, it has been described that the α-helix stabilizing function decreases in the order of norleucine > isoleucine [57]. Moreover, isoleucine can induce β-conformation stabilized by strong hydrophobic interactions [58–60]. It can be speculated that the higher α-helix stabilizing function of norleucine directly influences the binding affinity of the peptide **3a**. In addition, the peptide with the highest Y_1-receptor affinity, peptide **1a**, exhibited the most potent receptor selectivity. The strong Y_1-receptor selectivity is an important advantage compared to other known small Y_1-receptor binding peptides. GR231118 has also been characterized as Y_4-receptor agonist, which is a disadvantage in any peripheral studies [34–36]. Interestingly, also peptides **2a** and **3a** were found to be mainly Y_1-receptor selective with only weak Y_4-receptor affinities.

Small Y_1-receptor selective peptides are not only important tools for the investigation of the biological effects of NPY and its receptors but are also potential tumour markers since the Y_1-receptor over-expression has been determined in human breast cancer [16]. The most important disadvantage of peptides in pharmaceutical applications is their fast proteolytic degradation into small inactive fragments by, e.g. peptidases. In addition, several strategies are known to improve the metabolic stability of peptides, such as the association to a stabilizing protein, cyclization or the introduction of non-proteinogenic amino acids. NPY itself contains an amidated *C*-terminus that also enhances the metabolic stability. Accordingly, we determined the proteolytic degradation of peptides **1b, 2b** and **3b** in human blood plasma. Peptides were *N*-terminally labelled with CF to increase detection sensitivity. Peptides were found to be enzymatically four- to fivefold less stable (half-lives of 4.9 $\pm$ 0.3 h for peptide **1b**, 4.8 $\pm$ 1.6 h for peptide **2b** and 5.6 $\pm$ 3.5 h for peptide **3b**) than NPY (half-life of 24.7 $\pm$ 1.4 h). These results could be explained by the strong reduced size of the peptides. Peptides with fewer than ten amino acids are usually not able to form well-defined secondary structures resulting in a faster proteolytic degradation [61]. To characterize the degradation pattern of the peptides we determined their cleavage products. Peptides **1b, 2b** and **3b** were degradated at the *N*-terminal sites of the arginine33 as well as the arginine35. These results suggest a proteolytic degradation by amino peptidases. Previous studies of *full-length* NPY showed a similar degradation pattern with NPY(1–32) and NPY(1–34) as major cleavage products [62].

The most promising NPY analogue, peptide **1a**, was further investigated for Y_1-receptor activation by a signal transduction assay. Our results confirmed the nanomolar binding affinity at Y_1-receptors obtained in receptor binding affinity assays (EC_{50} value of 65.7 $\pm$ 8.9 nM). In addition, we found an agonistic activity of peptide **1a**. To further investigate the internalization property of peptide **1a** we used fluorescence microscopy. The results showed a clear internalization of the fluorescently labelled Y_1-receptor with only a faint membrane bound portion of the receptor left over after incubation. The slower internalization rate of peptide **1a** compared to NPY can be explained by the reduced receptor affinity and activation of the NPY analogue. This also suggests that a longer incubation time could lead to a complete peptide **1a**-mediated Y_1-receptor internalization.

Our SAR study identified three NPY analogues, peptides **1a, 2a** and **3a** with strong Y_1-receptor affinities and selectivities. In addition, in human blood plasma peptides were stable for several hours and enzymatically degradated at the arginine33 and the arginine35. Interestingly, the most promising peptide **1a** exhibited a strong agonistic activity at Y_1-receptors making this peptide to a useful tool in tumour targeting. Accordingly, for the first time, we developed a small peptidic Y_1-receptor selective agonist, which is a promising candidate to target breast cancer tumour cells.

Acknowledgements

The authors thank Regina Reppich-Sacher for mass spectrometry, Kristin Löbner, Doris Haines and Christina Damman for their technical help. This work was financially supported by the DFG (FOR630/BE 1264-9 and Graduate School BuildMoNa).

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