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Argininamide-type neuropeptide Y Y₁ receptor antagonists: the nature of N^ω-carbamoyl substituents determines Y₁R binding mode and affinity†

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The recently resolved crystal structure of the neuropeptide Y Y₁ receptor (Y₁R), co-crystallized with the high-affinity (pK_i: 10.11), argininamide-type Y₁R antagonist UR-MK299 (**2**), revealed that the N^ω-carbamoyl substituent (van der Waals volume: 139 Å³) is deeply buried in the receptor, occupying a hydrophobic pocket. We synthesized and characterized a series of argininamides, structurally related to **2**. Y₁R affinity decreased with increasing size of the carbamoyl residue (minimal pK_i: 5.67). Exceeding a critical size of the substituent (van der Waals volume: 212 Å³), the ligands bound in an inverted mode with the carbamoyl side chain located at the surface of the receptor, as suggested by induced-fit docking and MD simulations.

Introduction

Neuropeptide Y (NPY) receptors belong to the class A of G-protein coupled receptors (GPCRs).¹ The four functionally expressed subtypes in humans (Y₁R, Y₂R, Y₄R and Y₅R) are distributed in the central nervous system and in the periphery.² They are activated by the endogenous peptides neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP). The NPY Y₁R was shown to be overexpressed in different types of cancer (e.g. breast cancer).^{3,4} Therefore, labeled Y₁R ligands were suggested as potential tumor imaging agents.⁵ The first described non-peptide and selective Y₁R antagonist BIBP3226 (**1**, Table 1) binds in the low nanomolar range.⁶ Carbamoylation of the guanidine moiety of **1** led to UR-MK299 (**2**, Table 1), a Y₁R antagonist with picomolar affinity,⁷ which was recently co-crystallized with the NPY Y₁R.⁸ In the crystal structure, the carbamoylguanidine group of **2** forms a hydrogen-assisted salt bridge with D287^{6,59} and the propionyl group of the carbamoyl residue is buried in the subpocket between TM V and VI, which appears not to be completely filled (Fig. 1).⁸ This seems to be in agreement with

high Y₁R affinity (albeit lower compared to **2**) of (R)-argininamides with slightly larger carbamoyl residues as in **2**, such as compounds **3–5**, (Table 1).^{7,9} However, the identified binding mode of **2** doesn't seem to be compatible with the attachment of very bulky groups, such as fluorophores (**7–8**) or different carbamoyl residues (**9**), to the guanidine group of **1**, as suggested by low Y₁R affinities of **7–9** (Table 1). Therefore, reported fluorescent argininamide-type Y₁R ligands (labelled *via* N^ω-carbamoyl residues), exhibiting K_i values (Y₁R) between 20 and 150 nM,¹⁰ are supposed to bind to the Y₁R in a different manner compared to **2**.

In order to address the question how size and structure of the carbamoyl residue of N^ω-carbamoylated argininamides, structurally related to **2** and **3**, effect Y₁R affinity and the binding mode, we synthesized and pharmacologically characterized (competition binding and functional studies at the Y₁R) a series of N^ω-carbamoylated argininamides bearing carbamoyl residues of different sizes. The Y₁R binding mode of selected compounds was studied by induced-fit docking and molecular dynamics (MD) simulations.

Results and discussion

Synthesis

The N^ω-carbamoylated (R)-argininamides **53–76** and **78** were prepared as follows (note: the assignment of the numbers of target compounds **53–76** and **78** was guided by the size of the carbamoyl residue (*cf.* Table 2) and not by their synthetic accessibility outlined in Scheme 1): the carboxylic acids **10–20** were transformed to the respective succinimidyl esters **23–33** in the presence of DCC, and trifluoroacetic acid

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† Electronic supplementary information (ESI) available: Figures S1 and S2, Table S1, synthesis protocols and analytical data of compounds **23–34**, **38–39**, **41–42**, **53–76** and **78**, ¹H-NMR und ¹³C-NMR spectra of compounds **53–76**, RP-HPLC Purity Chromatograms of compounds **53–76** and **78**, investigation of the chemical stability of compounds **56**, **58–61**, **63** and **68**. See DOI: 10.1039/c9md00538b

Table 1 Structures and Y₁R affinities of the reported argininamides 1–9

Compd.	Ref.	R	pK _i
1 (BIBP-3226)	a, b		9.00
2 (UR-MK299)	a		10.11
3 (UR-MK136)	a, c		8.92
4	a		9.25
5	a		8.16
6	a		7.39
7	d		6.82
8	a		<6.00
9	a		6.40

References: a) Keller *et al.*,⁷ b) Rudolf *et al.*,⁶ c) Keller *et al.*,⁹ d) Keller *et al.*¹⁰ Reported K_i values were converted to pK_i values.

anhydride (**21**) was treated with *N*-hydroxysuccinimide (**22**) to obtain succinimidyl ester **34** (Scheme 1). Treatment of *tert*-butyl(2-aminoethyl)carbamate (**36**) or *tert*-butyl(3-aminopropyl)carbamate (**37**) with triphosgene gave isocyanates as intermediates, which were converted to the isothiourea derivatives **38** and **39** by the addition of **35** to the reaction mixture. The amine-functionalized argininamides **41** and **42** were obtained by guanidinylation of amine **40** using **38** and **39**, respectively, and subsequent removal of the Boc and *tert*-butyl groups by treatment with TFA (Scheme 1).

The target compounds **53–55**, **58** and **66–76** were synthesized by treatment of amines **41** or **42** with the succinimidyl esters **23–24**, **26–34** or **43–45** (Scheme 1). Compounds **56** and **57** were synthesized by amide bond formation between **41** and the carboxylic acids **46** and **47**, respectively, using DCC as coupling reagent (Scheme 1). Compounds **59–60** and **63–65** were synthesized from **41** and the carboxylic acids **48–52** according to the same procedure, but without the addition of DIPEA. Compound **61** was obtained by acylation of **41** using **25** and

Fig. 1 Extended view of the orthosteric binding pocket of the Y₁R occupied by **2** (ball and stick representation) (PDB ID: 5ZBQ⁸). The carbamoyl residue of **2** is sitting in a subpocket (oval area), located between TM V and VI. The crystal structure was refined (addition of hydrogen atoms, minimization, etc.; see Experimental section).

subsequent deprotection (Scheme 1). Alcohol **62** was isolated as degradation product of **60** after 6 months of storage of a 10 mM solution of **60** in DMSO at –20 °C. Compound **78** was synthesized by coupling of **41** with the pyrylium dye Py-5^{11,12} (**77**) according to a procedure reported previously by Keller *et al.*¹⁰ Chemical stabilities of compounds **56**, **58–61**, **63** and **68** were proven in aqueous solution, pH 7.4, at room temperature over 24 h (*cf.* ESI†).

Competition binding studies

Results from Y₁R competition binding experiments, performed at intact SK-N-MC cells using [³H]**2** as radioligand, are summarized in Table 2. Elongation of the ethylene spacer in **2** or **53** by one methylene group (**55** or **54**, respectively) did not affect Y₁R affinity. By contrast, elongation by two methylene groups (**3**) was reported to result in an approximately 30-fold decrease in affinity.⁹ The replacement of the propionyl group in **2** by mono- (**56**, **59–60**, **64–65**), di- (**57**) or tri- (**58**) halogenated acetyl or propionyl residues, as well as by amino (**61**) or hydroxy (**62**) functionalized acetyl residues did not significantly affect Y₁R affinity. Whereas the introduction of an acryl (**63**) or 2-methylpropionyl (**66**) residue followed the same trend, the more bulky 2,2-dimethylpropionyl residue in compound **67** led to an around 1000-fold decrease in Y₁R affinity compared to **2** (Table 2). In the series of compounds bearing aliphatic rings of increasing size (from cyclopropane to cyclohexane, **68–71**), Y₁R affinity considerably decreased (up to 5000-fold compared to **2**) in case of the cyclopentyl (**70**) and cyclohexyl (**71**) groups (Tables 1 and 2; competition binding

Table 2 γ_1 receptor affinities (pK_i) and antagonism (pK_b) of the synthesized N^{ω} -carbamoylated argininamides, determined by equilibrium competition binding with [3 H]2 and in the Fura-2 Ca^{2+} assay, respectively

Structure of **78** see Scheme 1.

Compd.	<i>n</i>	R	$pK_i \pm SEM^a$	$pIC_{50} \pm SEM/pK_b \pm SEM^b$
53	1		10.23 ± 0.06	n.d.
54	2		10.20 ± 0.05	n.d.
55	2		10.13 ± 0.04	n.d.
56	1		10.50 ± 0.04	$10.26 \pm 0.04/-$
57	1		10.17 ± 0.07	$9.96 \pm 0.12/-$
58	1		10.15 ± 0.06	$9.90 \pm 0.05/-$
59	1		10.28 ± 0.04	$10.10 \pm 0.04/-$
60	1		9.90 ± 0.07	$10.21 \pm 0.01/11.08 \pm 0.01$
61	1		9.62 ± 0.03	$9.55 \pm 0.03/10.43 \pm 0.03$
62	1		9.84 ± 0.05	$10.04 \pm 0.10/10.92 \pm 0.10$
63	1		9.95 ± 0.07	$9.73 \pm 0.05/10.61 \pm 0.05$
64	1		9.42 ± 0.05	$9.23 \pm 0.08/-$
65	1		9.16 ± 0.10	$9.39 \pm 0.10/10.27 \pm 0.10$
66	1		9.51 ± 0.09	$10.18 \pm 0.12/11.06 \pm 0.12$
67	1		7.34 ± 0.11	$7.84 \pm 0.08/8.71 \pm 0.08$
68	1		8.93 ± 0.12	$9.62 \pm 0.10/10.50 \pm 0.10$
69	1		8.96 ± 0.05	$9.62 \pm 0.09/10.49 \pm 0.09$
70	1		7.28 ± 0.07	$8.12 \pm 0.15/9.00 \pm 0.15$
71	1		6.42 ± 0.07	$7.51 \pm 0.01/8.39 \pm 0.01$
72	1		5.67 ± 0.05	$6.34 \pm 0.18/7.22 \pm 0.18$
73	1		7.25 ± 0.11	$7.94 \pm 0.03/8.82 \pm 0.03$
74	1		6.53 ± 0.07	$7.79 \pm 0.02/8.67 \pm 0.02$
75	1		6.52 ± 0.06	$7.02 \pm 0.04/7.90 \pm 0.04$
76	1		6.53 ± 0.01	$6.28 \pm 0.20/7.16 \pm 0.20$
78	1	n.a.	6.99 ± 0.04	n.d.

^a Radioligand competition binding assay with [3 H]2 ($c = 0.15$ nM, $K_d = 0.044$ nM⁷) at intact SK-N-MC cells. Mean values $\pm$ SEM from at least three independent experiments performed in triplicate. ^b Antagonistic activities determined in a Fura-2 Ca^{2+} assay at intact HEL cells.^{13,14} Intracellular Ca^{2+} mobilization was induced by 10 nM pNPY after preincubation of the cells with the antagonist for 15 minutes.⁷ pK_b values are not given, if the slope factor of the inhibition curve (four parameter logistic fit) was significantly different from ($P \leq 0.05$, see Table S1†) and not close (≤ 1.25 or ≥ 0.75) to unity, as a steep slope factor might be indicative of a more complex interaction not purely following the law of mass action. Mean values $\pm$ SEM from at least three independent experiments performed in singlet. n.d.: not determined. n.a.: not applicable.

Scheme 1 Synthesis of the N^{ω} -carbamoylated (*R*)-argininamides 53–76 and 78. Reagents and conditions: (a) DCC, CH_2Cl_2 , THF or DMF, 30–86%; (b) THF, 100%; (c) triphosgene, DIPEA, 50–71%; (d) (1) CH_2Cl_2 , HgCl_2 , DIPEA, (2) TFA/ CH_2Cl_2 1:1, 45–68%; (e) DIPEA, DMF, 21–84%; (f) DCC, DIPEA, DMF, 16–29%; (g) DCC, DMF, 9–16%; (h) (1) DIPEA, DMF, (2) CH_2Cl_2 /TFA 1:1, 46%; (i) DMSO; (j) DIPEA, DMF, 22%.

curves shown in Fig. S1A†). The insertion of a methylene group between the aliphatic ring and the amide group in 71, resulting in 72, even led to a further decrease in Y_1R affinity (>20 000-fold compared to 2; Tables 1 and 2 and Fig. S1A†). Interestingly, replacement of the aliphatic rings in 71 and 72 by a phenyl moiety (73 and 75) resulted in an approx. 10-fold increase in Y_1R affinity (Table 2, Fig. S1C†). Surprisingly, the introduction of a second benzene ring in 75, leading to 76, did not alter binding affinity, and, moreover, the introduction of a bulky pyridinium-type fluorescent dye (78) even resulted in a slightly higher affinity compared to 75/76 (Table 2, Fig. S1C†).

Functional studies

Y_1R antagonism (pK_b values) of 56–76, determined in a Fura-2 Ca^{2+} assay at HEL cells (inhibition of the intracellular Ca^{2+}

mobilization induced by pNPY), reflected the trends observed in competition binding studies. However, the pK_b values proved to be consistently slightly higher than the pK_i values (Table 2; inhibition curves shown in Fig. S1B and D†). Worth mentioning, modification of the carbamoyl substituents did not affect the mode of action of the Y_1R ligands, *i.e.* all compounds behaved as neutral antagonists.

Correlation of pK_i values with van der Waals volumes of the carbamoyl residues

For compounds 2–6 and 53–75, the experimentally determined pK_i values and the calculated van der Waals volumes of the respective carbamoyl residues showed an inverse correlation ($R^2 = 0.84$) between the size of the carbamoyl residue and the Y_1R affinity of the respective argininamide-type Y_1R antagonists (Fig. 2). By contrast, both, 1, which is unsubstituted at the guanidine group, and compounds bearing large carbamoyl residues (7, 9, 76, 78), appeared to be outliers in the regression analysis (Fig. 2). For 1, a much higher pK_i value would have been expected, and for 7, 9, 76 and 78 much lower values (*cf.* Fig. 2). Consequently, the attachment of small carbamoyl residues to the guanidine moiety (N^{ω}) of 1 (see compounds 2, 4, 53–66) led to a significant (up to more than one order of magnitude) increase in Y_1R affinity (Tables 1 and 2 and Fig. 2). By contrast, increasing van der Waals volumes of the carbamoyl

Fig. 2 Correlation between the experimentally determined ligand (2–6 and 53–75) pK_i values and calculated van der Waals volumes of the respective carbamoyl residues. Two types of outliers (squares) were observed: (1) argininamide 1, devoid of a carbamoyl substituent, supposed to bind in the same orientation as 2, but unable to occupy the subpocket between TM V and VI (*cf.* Fig. 1); (2) compounds 7, 9, 76 and 78, bearing bulkier carbamoyl moieties than 72, considered to bind to the Y_1R in a totally different orientation compared to 2.

Fig. 3 Cluster 1 binding poses of MD simulations (2 μ s) of the Y₁R (inactive state, PDB ID: 5ZBQ⁸) bound to 1 (A and B, orange), 2 (D and E, grey) or 3 (G and H, purple), and lowest free energy (MM-GBSA) conformations of 68 (F, blue), 72 (I, cyan), 76 (J and K, green) and 78 (L, magenta) obtained by induced-fit docking to the Y₁R. (C) Arginamide core structure. In A, D, G and J, the space within the subpocket between TM V and VI of the orthosteric binding pocket is highlighted with a blue surface/mesh illustration. Amino acids involved in H-bonding or salt bridges (indicated as yellow dashed lines), π - π interactions (green dashed lines) or cation- π interactions (magenta dashed lines) with the ligands are labeled: Y100^{2,64} (π - π): in B; Y100^{2,64} (HB): in E, F and K; F173^{4,60} (π - π): in K; Q177^{ECL2} (HB): in F and I; F199^{ECL2} (π - π): in F; D200^{ECL2} (HB, SB): in E, F, H, K and L; F202^{ECL2} (CAT- π): in E; T212^{5,39} (HB): in I and K; Q219^{5,46} (HB): in L; N283^{6,55} (HB): in B, F, I, K and L; T284^{6,56} (HB): in F; F286^{6,58} (π - π): in B; D287^{6,59} (HB, SB): in B, E, F, H, K and L; D287^{6,59} (HB): in I; N299^{7,32} (HB): in F, H and I; F302^{7,35} (π - π): in H. Amino acids involved in intra-molecular H-bonding or salt bridges (indicated as yellow dashed lines) are labeled: in B, R208^{ECL2}-D287^{6,59} (HB, SB); in E, D200^{ECL2}-R208^{ECL2} (HB, SB), T212^{5,39}-D287^{6,59} (HB). HB = hydrogen bond. SB = salt bridge. CAT = cation.

residues (see compounds 3, 5–6, 67–75; Tables 1 and 2 and Fig. 2) affected Y₁R binding. However, exceeding a critical volume (212 Å³) of the carbamoyl substituent (in compound 72), Y₁R affinity did not further decrease, but even increased (compounds 7, 9, 76 and 78; Tables 1 and 2 and Fig. 2). In order to find a molecular explanation for this phenomenon, computational studies were performed.

Induced-fit docking and MD simulations

To shed light on the binding modes of the most striking compounds (1–3, 68, 72, 76, 78) and to get insight into the molecular interactions leading to differences in Y₁R affinities, we performed MD simulations (1–3) and induced-fit docking (68, 72, 76, 78) (Fig. 3). All compounds showed the favorable hydrogen-assisted salt bridge (1–3, 68, 76, 78) or hydrogen bond (72) between the carbamoylguanidine moiety and D287^{6.59} in cluster 1 of the MD simulations (1–3) or the lowest free energy (MM-GBSA score) binding poses of induced-fit docking (68, 72, 76, 78) (Fig. 3B, E, F, H, I, K and L). Noteworthy, when comparing the Y₁R affinity of 5 with its congener 6 (methylated at the carbamoyl nitrogen, see Table 1), it becomes obvious that the carbamoyl N–H group is involved in binding. In addition to the interaction with D287^{6.59}, the carbamoylguanidine moiety of most compounds (2, 3, 68, 76, 78) simultaneously formed a hydrogen-assisted salt bridge with D200^{ECL2} (Fig. 3E, F, H, K and L). By contrast, the guanidine moiety of 1 was either in contact with D287^{6.59} or D200^{ECL2} in the MD simulation (Fig. S2A†). Interestingly, in addition to the interaction with the carbamoylguanidine moiety of the ligands, D200^{ECL2} showed an intra-molecular salt bridge with R208^{ECL2}, which was most pronounced in case of 2 (Fig. 3E).

Further specific (non-hydrophobic) interactions between amino acids of the Y₁R and the ligands were hydrogen bonds (Y100^{2.64} (2, 68, 76), Q177^{ECL2} (68, 72), T212^{5.39} (2, 72, 76), Q219^{5.46} (78), N283^{6.55} (1, 68, 72, 76, 78), T284^{6.56} (68), N299^{7.32} (3, 68, 72)), π – π (Y100^{2.64} (1), F173^{4.60} (76), F199^{ECL2} (68), F286^{6.58} (1), F302^{7.35} (3)) or cation– π contacts (F202^{ECL2} (2)) (Fig. 3).

In MD simulations, reference compound 1, bearing no carbamoyl substituent at the guanidine group, showed a binding mode (Fig. 3A and B) comparable to that of 2 (Fig. 3D and E). However, in contrast to 2, 3, 68 and 72, argininamide 1 did not occupy the subpocket between TM V and VI due to the lacking residue at the guanidine group (Fig. 3A, B and D–I).

Whereas an extension of the ethylene spacer in 2 by one methylene group (55) did not decrease Y₁R affinity (pK_i of 2 and 55: 10.11 and 10.13, respectively), an extension by two methylene groups, resulting in 3, led to a significant decrease in Y₁R binding by more than one order of magnitude (pK_i of 3: 8.92). This demonstrated that the carbamoyl residue in 3 is already too large for an ideal occupation of the subpocket, which is reflected by an upward movement (towards the receptor surface) of the ligand core structure (*cf.* Fig. 3C) during the MD simulation (Fig. 3G and H and S2C†).

For compounds belonging to the aliphatic ring series (68–72, *cf.* Table 2), the decrease in Y₁R affinity observed for ring sizes > four carbon atoms (70–72) might be explained by an increasing distance between the carbamoylguanidine moiety and D287^{6.59} preventing salt bridge formation in case of 72 (Fig. 3I). Therefore, compounds bearing bulkier carbamoyl substituents compared to 72 (7, 9, 76, 78) would be expected to exhibit lower Y₁R affinities than 72 (Tables 1 and 2). However, the opposite was the case, *i.e.* an increase in affinity was observed. A possible explanation for this phenomenon could be an inverted binding mode, as identified for 76 and 78 by induced-fit docking: the diphenyl acetyl and 4-hydroxybenzyl moieties of 76 and 78 were found to be deeply buried in the orthosteric binding pocket, with the 4-hydroxybenzyl group sitting in the subpocket between TM V and VI (Fig. 3J–L). As a consequence, the carbamoyl residues of 76 and 78 pointed to the cytoplasmic side (Fig. 3J–L). Strikingly, the suggested inverted binding mode of 76 and 78 was accompanied by a reconstitution of the crucial interaction of the carbamoylguanidine moiety with D200^{ECL2} and D287^{6.59}, explaining the gain in Y₁R affinity.

Conclusions

We synthesized and characterized a series of argininamide-type Y₁R antagonists, bearing different carbamoyl residues (small (53–69) *vs.* bulky (70–76 and 78), cyclic (68–76 and 78) *vs.* acyclic (53–67)) at the guanidine group. Up to a critical size of the carbamoyl side chain (*e.g.* compound 72), the increase in size correlated inversely with Y₁R affinity (pK_i values: 5.67–10.50), showing that the van der Waals volume of considerably larger carbamoyl substituents than in reference compound 2 is too large to allow the occupation of the subpocket located between TM V and TM VI. As suggested by induced-fit docking and MD simulations, argininamides bearing very bulky carbamoyl residues (*e.g.* fluorescent ligand 78) bind in an inverted mode (compared to 2), accompanied by a moderate recovery of Y₁R affinity (pK_i of 78: 6.99). The present study revealed that the subpocket of the Y₁R, perfectly occupied by the carbamoyl residue of the high affinity Y₁R antagonist 2,⁸ cannot harbor large moieties such as fluorescent dyes. In view of the preparation of future high affinity fluorescent ligands for the Y₁R derived from 2, the label should not be attached to the carbamoyl residue, but to a different, more favorable site such as the diphenyl acetyl moiety pointing to the receptor surface.

Experimental

General experimental conditions

In general, all used reagents and solvents (analytical grade) were purchased from commercial suppliers and used without further purification: CH₂Cl₂, DMF (Fisher Scientific, Schwerte, Germany); DCC, TFA, 10–11, 13–20, 22 and 46–51 (Sigma Aldrich, München, Germany); triphosgene and 21

(TCI, Eschborn, Germany); DIPEA, **36** (Abcr, Karlsruhe, Germany); **52** (Merck, Darmstadt, Germany).

Compounds **12**,¹⁵ **35**,¹⁴ **37**,¹⁶ **40**,¹⁷ **44**,¹⁸ **45**¹⁹ and **77**^{11,12} were synthesized as described previously. Column chromatography was performed using Merck Gerduran 60 silica gel (0.063–0.200 mm) or Merck flash silica gel 60 (0.040–0.063 mm). For thin layer chromatography, TLC sheets ALUGRAM Xtra SIL G/UV254 from Macherey-Nagel GmbH & Co. KG (Düren, Germany) were used. UV spots were detected by irradiation with UV light (254 nm), and staining was performed with ninhydrin.

Acetonitrile (HPLC grade) used for HPLC was purchased from Sigma-Aldrich. Millipore water was used for eluents of analytical and preparative HPLC. Compounds **41–42**, **53–76** and **78** were purified by a preparative HPLC-system from Knauer (Berlin, Germany) consisting of two pumps (K-1800) and a detector (K-2001). A Kinetex XB C18, 5 μ m, 250 $\times$ 21 mm (Phenomenex, Aschaffenburg, Germany) served as RP-column at a flow rate of 18 mL min⁻¹. All injection solutions were filtrated with syringe filters (0.45 μ m). The mobile phase contained the solvents A (0.1% aq TFA) and B (acetonitrile). The detection wavelength was 220 nm. Acetonitrile was removed from the eluates at 40 °C under reduced pressure. The eluate was lyophilized using a Christ alpha 2-4 LD (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) or a Scanvac CoolSafe 100-9 (Labogene, Allerød, Denmark) lyophilization apparatus equipped with a Vacuubrand RZ rotary vane vacuum pump (Vacuubrand, Wertheim, Germany).

The purity of compounds **53–65**, **73** and **78** was determined by analytical HPLC (RP-HPLC) on an 1100 series system from Agilent Technologies (Santa Clara, CA USA) composed of a Degasser (G1379A), a Binary Pump (G1312A), a Diode Array Detector (G1315A), a thermostated Column Compartment (G1316A) and an Autosampler (G1329A). A Phenomenex Kinetex 5u XB-C18 100A, 250 $\times$ 4.6 mm was used as stationary phase. The flowrate was 1 mL min⁻¹, the oven temperature was set to 30 °C and the injection volume was 50 μ L. Mixtures of solvents A (0.5% aq TFA) and B (acetonitrile) were used as mobile phase. The following gradient was applied: method A: 0–25 min, A/B 90:10–5:95; 25–35 min, 5:95. Analytical HPLC analysis of compounds **66–72** and **74–76** was performed on a system from Merck-Hitachi composed of a Pump (L-6200A), an Interface (D600 IF), an Autosampler (AS-2000) and an UV-Detector (L-4000A). A Phenomenex Kinetex 5u XB-C18 100A, 250 $\times$ 4.6 mm (Phenomenex) was used as stationary phase. The flowrate was 0.8 mL min⁻¹, the oven temperature was set to 30 °C and the injection volume was 35 μ L. A mixture of solvents A (0.5% aq TFA) and B (0.5% TFA, acetonitrile) was used as mobile phase. The following gradients were applied: method B: 0–25 min, A/B 90:10–5:95; 25–35 min, 5:95 and method C: 0–30 min, 95:5–20:80; 30–32 min, 20:80–5:95; 32–42 min, 5:95. Purity was detected at 220 nm. The retention factor *k* was calculated according to following equation: $k = (t_R - t_0)/t_0$ (t_0 = dead time).

NMR spectra were recorded on a Bruker Avance 300 (¹H, 300 MHz, ¹³C, 75 MHz), a Bruker Avance III 400 (¹H, 400 MHz, ¹³C, 101 MHz) and a Bruker Avance 600 with cryogenic probe (¹H, 600 MHz; ¹³C, 150 MHz) (Bruker, Karlsruhe, Germany). Chemical shifts are given in ppm and were referenced to the solvent residual peak (DMSO-*d*₆, at 2.50 ppm and at 39.52 ppm (¹³C-NMR); CDCl₃, at 7.26 ppm (¹H-NMR) and at 77.16 ppm (¹³C-NMR)).²⁰ The coupling constants (*J*) are given in Hertz (Hz). The splitting of the signals is described as follows: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

Mass spectrometry (HRMS) analysis was performed either on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies) using an electrospray source (ESI) or on an Agilent GC7890A GC/MS system (Agilent Technologies) using an atmospheric pressure chemical ionization (APCI).

Stock solutions were prepared in DMSO at concentrations of 2 mM or 10 mM.

General synthesis procedures

General procedure A. The precursors **41** or **42** were dissolved in DMF, and DIPEA was added. The succinimidyl esters **23–33**, **43–45**, except **34**, were dissolved in DMF and added to the solution of **41** or **42**. The reaction mixture was stirred at rt for 1–2 h. 10% aq TFA (10 equiv.) were added and the product was isolated by preparative HPLC.

General procedure B. A freshly prepared solution of the carboxylic acids **48–52** and DCC in DMF (0.5 mL) was added dropwise to a solution of **41** in DMF (1 mL) and the mixture was stirred at rt for 2–3 h. A solid material was removed by filtration and the product purified by preparative HPLC.

General procedure C. In contrast to general procedure B, the carboxylic acids **46–47** were used and DIPEA (2.5 equiv.) was added to the solution of **41** in DMF.

Compound characterization

Target compounds (**53–76**, **78**) were characterized by ¹H NMR, ¹³C NMR and 2D NMR (HSQC, HMBC) spectroscopy, HRMS and RP-HPLC analysis. The purity (HPLC, 220 nm) of all target compounds amounted to $\geq 95\%$ (chromatograms shown in the ESI†).

Screening for Pan-assay interference compounds (PAINS)

Screening of all target compounds for PAINS *via* the public tool <http://zinc15.docking.org/patterns/home>²¹ gave no hits except for compound **78** (*N,N*-dimethylaniline partial structure was identified as PAIN). The identity of **78** was proven by HRMS and the compound exhibited a purity of 95%. Moreover, there are no reports on the *N,N*-dimethylaniline scaffold to exhibit Y₁R affinity as determined for **78**. Therefore, an interference in the radioligand competition binding assay by an impurity containing a *N,N*-dimethylaniline scaffold can be excluded.

Radioligand binding assay

All competition binding experiments at the Y₁R were essentially performed as described by Keller *et al.*⁷ using [³H]2 (*c* = 0.15 nM) and SK-N-MC cells expressing the Y₁R. Three independent experiments were performed in triplicate. Specific binding data were plotted as % (100% = bound radioligand in the absence of competitor) over log(concentration competitor) and analyzed by four-parameter logistic fits (GraphPad Prism 8.0, GraphPad, San Diego, CA USA) to obtain pIC₅₀ values, which were converted to pK_i values according to the Cheng-Prusoff equation²² (logarithmic form) (used K_d value of [³H]2: 0.044 nM⁷).

Fura-2 calcium assay

The Fura-2 Ca²⁺ assay at the Y₁R was essentially performed as described by Müller *et al.*¹³ using 10 nM pNPY for intracellular Ca²⁺ mobilization and applying a pre-incubation period of 15 min for the antagonists. Three independent experiments were performed in singlet. Relative Ca²⁺ responses were plotted as % against log(concentration antagonist) and analyzed by four-parameter logistic fits (GraphPad Prism version 8.0) to obtain pIC₅₀ values, which were converted to pK_b values according to the Cheng-Prusoff equation²² (logarithmic form) (used EC₅₀ value of pNPY: 1.53 nM).

Computational chemistry

Receptor and ligand preparation. The crystal structure of the inactive state Y₁R bound to the antagonist 2 (PDB ID: 5ZBQ⁸) was used as template. Minor modifications were performed using the modeling suite SYBYL-X 2.0 (Tripos Inc., St. Louis, MO USA): the ICL3 loop was reconstituted by the wild-type sequence. Coordinates of nonligand and nonreceptor molecules were removed. Protein and ligand preparation (Schrödinger LLC, Portland, OR USA) including an assignment of protonation states were essentially performed as described in Pegoli *et al.*^{23,24} Disulfide bonds of the Y₁R were maintained between C33^{N-term} and C296^{7,29} as well as C113^{3,25} and C198^{45,50}, and a sodium ion was placed next to D86^{2,50}. Guanidine groups and the fluorophore Py-5 were singly protonated, resulting in a net charge of +1 for 1–3, 68, 72, 76, and +2 for the fluorescence ligand 78.

Induced-fit docking. “Flexible” docking of 1–3, 68, 72, 76 and 78 to the Y₁R was performed using the induced-fit docking module (Schrödinger LLC). The ligands were docked within a box of 46 × 46 × 46 Å³ around the crystallographic binding pose of 2. Redocking was performed in the extended precision mode. The resulting poses were scored using MM-GBSA (Schrödinger LLC). Among the reasonable ligand binding poses, the pose corresponding to the lowest MM-GBSA value was selected as the most probable pose. For compounds 1–3, the coordinates of this pose were used as input for subsequent MD simulations.

Molecular dynamics simulations. Simulations of the Y₁R bound to 1, 2 or 3 and trajectory analysis were essentially performed as described in Pegoli *et al.*²³ with the following

modifications: the docked ligand–receptor complexes were aligned to the NTS₁R entry (PDB ID: 4BUO²⁵) in the orientations of proteins in membranes (OPM) database.²⁶ The Desmond system builder within Maestro (Schrödinger LLC) was used to insert the ligand–receptor complexes into hydrated, equilibrated palmitoylcholinephosphatidylcholine (POPC) bilayers, comprising about 160 POPC molecules as well as sodium chloride at a concentration of 150 mM (net charges of the entire systems were zero). The systems contained about 78 000 atoms and the box sizes were approximately 81 × 87 × 117 Å³. The coordinates were successively converted to chamber topology and coordinate files using inhouse scripts, psfgen,²⁷ htmd²⁸ and chamber (AMBER 2016, University of California, San Francisco, CA USA). Ligand partial charges were further optimized using fftk.²⁹ After minimization, the systems were heated from 0 to 100 K in the NVT ensemble during 20 ps and from 100 to 310 K in the NPT ensemble during 100 ps, applying harmonic restraints of 5 kcal mol^{−1} Å^{−1} to non-hydrogen atoms of protein and ligand. During the equilibration period (10 ns), harmonic restraints on receptor and ligand non-hydrogen atoms were reduced stepwise (0.5 kcal mol^{−1} Å^{−1} every 0.5 ns) to 2.5 kcal mol^{−1} Å^{−1} within 3 ns. While removing restraints on ligand atoms, harmonic restraints on receptor mainchain atoms were further reduced stepwise (0.5 kcal mol^{−1} Å^{−1} every 0.5 ns) to 0.5 kcal mol^{−1} Å^{−1} from 3 to 5 ns. After 5 ns, harmonic restraints on receptor mainchain atoms were removed, *i.e.* the residual equilibration period (5 ns) was run without restraints. The interaction cutoff was set to 9.0 Å. The final frames of the equilibration period were used as input for the simulations over 2 μs. Ligand–receptor interactions were analyzed using PLIP 1.4.2.³⁰ Figures showing molecular structures of the Y₁R in complex with 1, 2, 3, 68, 72, 76 or 78 were generated with PyMOL Molecular Graphics system, version 2.2.0 (Schrödinger LLC).

Calculation of van der Waals volumes. ChemAxon Marvin Calculator Plugins (Marvin 18.24.0, 2018, ChemAxon, <http://www.chemaxon.com>) were used to calculate the van der Waals volumes of the respective carbamoyl residues (containing a radical at the carbonyl group) of compounds 1–7, 9, 53–76 and 78.

Author contributions

J. B. and T. S. performed the syntheses, analytical characterization as well as competition binding and functional experiments. D. W. performed molecular docking, MD simulations and processed the data. D. W., M. K. and G. B. supervised the research. J. B., D. W., M. K. and G. B. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- 1 M. C. Michel, A. Beck-Sickinger, H. Cox, H. N. Doods, H. Herzog, D. Larhammar, R. Quirion, T. Schwartz and T. Westfall, *Pharmacol. Rev.*, 1998, **50**, 143–150.
- 2 F. Reichmann and P. Holzer, *Neuropeptides*, 2016, **55**, 99–109.
- 3 L. Zhang, M. S. Bijker and H. Herzog, *Pharmacol. Ther.*, 2011, **131**, 91–113.
- 4 J. C. Reubi, M. Gugger, B. Waser and J. C. Schaer, *Cancer Res.*, 2001, **61**, 4636–4641.
- 5 J. Li, Y. Tian and A. Wu, *Regener. Biomater.*, 2015, **2**, 215–219.
- 6 K. Rudolf, W. Eberlein, W. Engel, H. A. Wieland, K. D. Willim, M. Entzeroth, W. Wienen, A. G. Beck-Sickinger and H. N. Doods, *Eur. J. Pharmacol.*, 1994, **271**, R11–R13.
- 7 M. Keller, S. Weiss, C. Hutzler, K. K. Kuhn, C. Mollereau, S. Dukorn, L. Schindler, G. Bernhardt, B. König and A. Buschauer, *J. Med. Chem.*, 2015, **58**, 8834–8849.
- 8 Z. Yang, S. Han, M. Keller, A. Kaiser, B. J. Bender, M. Bosse, K. Burkert, L. M. Kogler, D. Wifling, G. Bernhardt, N. Plank, T. Littmann, P. Schmidt, C. Yi, B. Li, S. Ye, R. Zhang, B. Xu, D. Larhammar, R. C. Stevens, D. Huster, J. Meiler, Q. Zhao, A. G. Beck-Sickinger, A. Buschauer and B. Wu, *Nature*, 2018, **556**, 520–524.
- 9 M. Keller, G. Bernhardt and A. Buschauer, *ChemMedChem*, 2011, **6**, 1566–1571.
- 10 M. Keller, D. Erdmann, N. Pop, N. Pluym, S. Teng, G. Bernhardt and A. Buschauer, *Bioorg. Med. Chem.*, 2011, **19**, 2859–2878.
- 11 B. K. Höfelschweiger, *PhD thesis*, University of Regensburg, 2005.
- 12 B. K. Wetzl, S. M. Yarmoluk, D. B. Craig and O. S. Wolfbeis, *Angew. Chem., Int. Ed.*, 2004, **43**, 5400–5402.
- 13 M. Müller, S. Knieps, K. Geßele, S. Dove, G. Bernhardt and A. Buschauer, *Arch. Pharm.*, 1997, **330**, 333–342.
- 14 S. Weiss, M. Keller, G. Bernhardt, A. Buschauer and B. König, *Bioorg. Med. Chem.*, 2008, **16**, 9858–9866.
- 15 N. Gavande, H.-L. Kim, M. R. Doddareddy, G. A. R. Johnston, M. Chebib and J. R. Hanrahan, *ACS Med. Chem. Lett.*, 2013, **4**, 402–407.
- 16 A. Montero, P. Goya, N. Jagerovic, L. F. Callado, J. J. Meana, R. O. Girón, C. Goicoechea and M. I. Martín, *Bioorg. Med. Chem.*, 2002, **10**, 1009–1018.
- 17 M. Keller, N. Pop, C. Hutzler, A. G. Beck-Sickinger, G. Bernhardt and A. Buschauer, *J. Med. Chem.*, 2008, **51**, 8168–8172.
- 18 R. Wodtke, C. Hauser, G. Ruiz-Gómez, E. Jäckel, D. Bauer, M. Lohse, A. Wong, J. Pufe, F.-A. Ludwig, S. Fischer, S. Hauser, D. Greif, M. T. Pisabarro, J. Pietzsch, M. Pietsch and R. Löser, *J. Med. Chem.*, 2018, **61**, 4528–4560.
- 19 S. Dissoki, A. Hagooley, S. Elmachily and E. Mishani, *J. Labelled Compd. Radiopharm.*, 2011, **54**, 693–701.
- 20 H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512–7515.
- 21 C. Aldrich, C. Bertozzi, G. I. Georg, L. Kiessling, C. Lindsley, D. Liotta, K. M. Merz, Jr., A. Schepartz and S. Wang, *J. Med. Chem.*, 2017, **60**, 2165–2168.
- 22 Y. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, 1973, **22**, 3099–3108.
- 23 A. Pegoli, X. She, D. Wifling, H. Hübner, G. Bernhardt, P. Gmeiner and M. Keller, *J. Med. Chem.*, 2017, **60**, 3314–3334.
- 24 A. Pegoli, D. Wifling, C. G. Gruber, X. She, H. Hübner, G. Bernhardt, P. Gmeiner and M. Keller, *J. Med. Chem.*, 2019, **62**, 5358–5369.
- 25 P. Egloff, M. Hillenbrand, C. Klenk, A. Batyuk, P. Heine, S. Balada, K. M. Schlinkmann, D. J. Scott, M. Schutz and A. Pluckthun, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, E655–E662.
- 26 M. A. Lomize, A. L. Lomize, I. D. Pogozheva and H. I. Mosberg, *Bioinformatics*, 2006, **22**, 623–625.
- 27 W. Humphrey, A. Dalke and K. Schulten, *J. Mol. Graphics*, 1996, **14**, 33–38, 27–38.
- 28 S. Doerr, M. J. Harvey, F. Noe and G. De Fabritiis, *J. Chem. Theory Comput.*, 2016, **12**, 1845–1852.
- 29 C. G. Mayne, J. Saam, K. Schulten, E. Tajkhorshid and J. C. Gumbart, *J. Comput. Chem.*, 2013, **34**, 2757–2770.
- 30 S. Salentin, S. Schreiber, V. J. Haupt, M. F. Adasme and M. Schroeder, *Nucleic Acids Res.*, 2015, **43**, W443–W447.