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Synthesis and biological activity of nociceptin/orphanin FQ analogues substituted in position 7 or 11 with Cα,α-dialkylated amino acids

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Abstract—Previous structure–activity and NMR studies on nociceptin/orphanin FQ (N/OFQ) demonstrated that Aib substitution of Ala[7] and/or Ala[11] increases the peptide potency through an alpha helix structure induction mechanism. On these bases we synthesised and evaluated pharmacologically in the mouse vas deferens assay a series of N/OFQ-NH_2 analogues substituted in position 7 and 11 with Cα,α-disubstituted cyclic, linear and branched amino acids. None of the 20 novel N/OFQ analogues produced better results than [Aib[7]]N/OFQ-NH_2. Thus, this substitution was combined with other chemical modifications known to modulate peptide potency and/or efficacy generating compound **21** [Nphe[1]Aib[7]Arg[14]Lys[15]]N/OFQ-NH_2 (coded as UFP-111), compound **22** [(pF)Phe[4]Aib[7]Arg[14]Lys[15]]N/OFQ-NH_2 (UFP-112) and compound **23** [Phe[1]Ψ(CH_2–NH)Gly[2](pF)Phe[4]Aib[7]Arg[14]Lys[15]]N/OFQ-NH_2 (UFP-113). These novel peptides behaved as highly potent NOP receptor ligands showing full (UFP-112) and partial (UFP-113) agonist and pure antagonist (UFP-111) activities in a series of in vitro functional assays performed on pharmacological preparations expressing native as well as recombinant NOP receptors.

1. Introduction

Nociceptin/orphanin FQ[1,2] (N/OFQ) is a neuropeptide characterized by the following primary sequence: H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH. N/OFQ modulates several biological functions at peripheral (respiratory, cardiovascular and renal, gastrointestinal, immune systems) and central (nociception, memory, food intake, locomotor activity, response to stress and anxiety) levels[3] by selectively activating an opioid-like receptor named N/OFQ peptide (NOP) receptor.[4]

Identification of novel NOP receptor ligands of either peptide or nonpeptide nature has been the subject of intense research activity.[5,6] Structure–activity studies on N/OFQ lead to the identification of chemical modifications useful for increasing peptide potency ([(pF)Phe[4]][7] and [Arg[14]Lys[15]] replacement[8]) or for reducing ([Phe[1]Ψ(CH_2–NH)Gly[2]][9,10]) or eliminating ([Nphe[1]] replacement[11]) efficacy at the NOP receptor. The combination in the N/OFQ-NH_2 sequence of the chemical modifications reported above produced a series of highly potent selective NOP receptor ligands.[12–14] The replacement of Ala[7], Ala[11] and Ala[15] with the Cα,α dimethylated amino acid Aib in N/OFQ sequence in the case of [Aib[7]]N/OFQ-NH_2 produced a NOP receptor ligand 7-fold more potent than the parent peptide as determined in the [^{35}S]GTPγS functional assay.[15] The higher potency of the Aib substituted analogues of N/OFQ has been ascribed by computational methods to a possible increase in the alpha helix structure of the C-terminal

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portion of the peptide.[15] NMR studies of Aib substituted N/OFQ analogues demonstrated the high propensity of these molecules to adopt alpha helical conformations.[16] Moreover, the replacement of Ala^7 and Ala^{11} residues with Leu (a natural amino acid residue that can impart helicity) or Pro (a residue that favours disruption of the helix) in the N/OFQ-NH_2 sequence generated N/OFQ analogues with similar and 100-fold decreased potency in comparison with N/OFQ, respectively. This corroborates the idea that the helical structure of the peptide favours NOP receptor interaction.[16]

NMR studies have demonstrated that N/OFQ shows random coil conformations in water[17] while in aqueous/TFE mixture N/OFQ displays some conformational preferences compatible with an alpha helix structure.[18] Moreover, N/OFQ exhibits considerably more stable helical structure from Ala^7 to Gln^{17} in the presence of SDS micelles,[18] a system often employed in NMR investigations to mimic membrane-like environments. The presence of an alpha helix in the N/OFQ C-terminal portion Arg^8-Arg^{13} was also suggested by circular dichroism, NMR spectroscopy and theoretical conformational analyses.[19]

Collectively these structural data suggested a pharmacophore model in which the C-terminal portion of N/OFQ is organized as an alpha helix with one face of the helix highly positively charged by the presence of the side chains of $Arg^{8,12}$ and $Lys^{9,13}$ This cluster of positive charges seems to play a crucial role for interacting with the second extracellular loop of the NOP receptor which is rich in acidic residues.[20] In this regard, it is worthy of mention that the distance of the positive charges from the peptide backbone is also important as recently demonstrated by the increase in N/OFQ(1–13)-NH_2 potency obtained with the Lys^9/Orn substitution.[21]

Here, we report the synthesis and biological evaluation in the mouse vas deferens assay[22] of a series of N/OFQ-NH_2 analogues substituted in position 7 and 11 with Cα,α-disubstituted cyclic, linear and branched amino acids (Chart 1) compatible with alpha helix structures. Moreover, we investigated in a battery of in vitro assays, the effects induced by combining in the N/OFQ-NH_2 template the chemical modifications that increase the ligand potency ([(pF)Phe^4] and [$Arg^{14}Lys^{15}$]), reduce ([$Phe^1\Psi(CH_2–NH)Gly^2$]) or eliminate ([$Nphe^1$]) ligand efficacy with that able to promote alpha helix structure ([Aib^7] replacement).

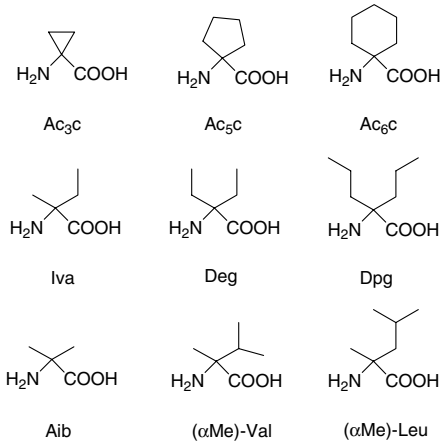


Chart 1. Chemical structures of the amino acids employed in this study.

2. Results and discussion

Peptides **1**–**23** reported in Table 1 were prepared with good yield by solid phase synthesis using HATU, an efficient peptide coupling reagent particularly indicated in the acylation steps of hindered amino acids.[23] The Cα,α-disubstituted amino acids Iva, Deg and Dpg were prepared with good yields following the Strecker procedure[24] using tetrabutylammonium cyanide instead of KCN (Scheme 1). This method involves the formation of a hydantoin from a ketone, followed by alkaline hydrolysis to the amino acid. The Fmoc protection[25] of the alpha amino function gave the protected amino acids Fmoc-Iva-OH, Fmoc-Deg-OH and Fmoc-Dpg-OH further employed in the solid phase peptide synthesis. For the synthesis of compounds **4**–**9** and **15**–**18** the amino acids (αMe)-Val, (αMe)-Leu and Iva have been employed as a racemic mixture. The corresponding diastereomers were fully separated in the case of N/OFQ analogues substituted in position 7, while in position 11 we were able to obtain a satisfactory separation of the two diastereomers only in the case of (αMe)-Val. Compounds **17** and **18** were evaluated pharmacologically as a diastereomeric mixture.

Compounds **1**–**20** in addition to the reference compounds N/OFQ-NH_2 [Aib^7]N/OFQ-NH_2, and [Aib^{11}]N/OFQ-NH_2 were evaluated pharmacologically using the electrically stimulated mouse vas deferens as a N/OFQ sensitive preparation.[22] The results of these studies are summarized in Table 2. All the N/OFQ-NH_2 analogues substituted in position 7 and 11 with Cα,α-disubstituted amino acids mimicked the inhibitory action elicited by N/OFQ-NH_2 displaying maximal effects superimposable to those of the reference peptide. In line with previous findings,[15,16] the replacement of Ala^7 and Ala^{11} with Aib produced an increase in peptide potency which was ~2-fold higher for the position 7 substituted analogue.

The use of Aib constrained cyclic derivatives Ac_3c, Ac_5c and Ac_6c (compounds **1**–**3**) in position 7 produced less potent analogues compared to [Aib^7]N/OFQ-NH_2. Similar results were obtained by the replacement of position 7 with the branched Aib analogues (αMe)-Val and (αMe)-Leu (compounds **4**–**7**). The use of the linear Aib analogues Iva, Deg and Dpg (Compounds **8**–**11**) produced peptides equipotent to the standard reference N/OFQ-NH_2 but still less potent than [Aib^7]N/OFQ-NH_2. All of these Aib analogues are compatible with alpha helix structures as suggested by a series of studies performed on both model[26,27] and bioactive[28] peptides. Thus, it is unlikely that the decrease in peptide potency

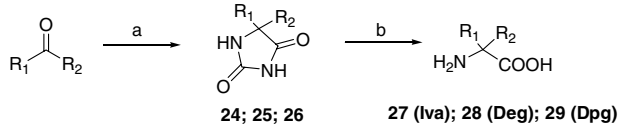
Table 1. Abbreviated names and analytical properties of the N/OFQ analogues

No.	Abbreviated names	[a]t_R		[b]MH^+	
		I	II	Calculated	Found
	N/OFQ-NH_2	10.53	8.77	1809.0	1809.2
	[Aib^7]N/OFQ-NH_2	10.62	9.88	1822.2	1822.4
	[Aib^{11}]N/OFQ-NH_2	11.08	10.28	1822.2	1822.6
1	[Ac_3c^7]N/OFQ-NH_2	9.80	8.63	1821.5	1821.7
2	[Ac_5c^7]N/OFQ-NH_2	11.78	10.93	1849.5	1849.9
3	[Ac_6c^7]N/OFQ-NH_2	12.26	11.45	1863.4	1863.7
4	[(αMe)D/L-Val^7]N/OFQ-NH_2(1)	11.00	11.39	1851.4	1851.2
5	[(αMe)D/L-Val^7]N/OFQ-NH_2(2)	11.84	11.44	1851.4	1851.2
6	[(αMe)D/L-Leu^7]N/OFQ-NH_2(1)	12.19	11.36	1865.5	1865.9
7	[(αMe)D/L-Leu^7]N/OFQ-NH_2(2)	12.64	11.46	1865.5	1865.9
8	[D/L-Iva^7]N/OFQ-NH_2(1)	11.14	10.03	1837.5	1837.6
9	[D/L-Iva^7]N/OFQ-NH_2(2)	11.20	10.51	1837.5	1837.6
10	[Deg^7]N/OFQ-NH_2	11.36	10.72	1850.8	1851.1
11	[Dpg^7]N/OFQ-NH_2	12.85	11.62	1878.6	1879.0
12	[Ac_3c^{11}]N/OFQ-NH_2	10.09	8.62	1821.5	1821.7
13	[Ac_5c^{11}]N/OFQ-NH_2	11.98	11.32	1849.5	1849.1
14	[Ac_6c^{11}]N/OFQ-NH_2	11.99	11.83	1863.4	1863.7
15	[(αMe)D/L-Val^{11}]N/OFQ-NH_2 (1)	11.42	10.78	1851.4	1851.7
16	[(αMe)D/L-Val^{11}]N/OFQ-NH_2 (2)	11.64	10.90	1851.4	1851.7
17	[(αMe)D/L-Leu^{11}]N/OFQ-NH_2	12.50	11.66	1865.5	1865.0
18	[D/L-Iva^{11}]N/OFQ-NH_2	11.40	10.92	1837.5	1837.9
19	[Deg^{11}]N/OFQ-NH_2	11.59	11.00	1850.8	1851.4
20	[Dpg^{11}]N/OFQ-NH_2	12.62	11.82	1878.6	1878.2
21	[Nphe^{1}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2	9.18	7.09	1923.0	1923.4
22	[(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2	9.85	7.89	1941.1	1941.4
23	[Phe^{1}Ψ(CH_2–NH)Gly^2(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2	9.38	6.65	1927.6	1927.9

(1) and (2) see Section 4.

[a] t_R is the retention time determined by analytical HPLC.

[b] The mass ion (MH^+) was obtained by electrospray mass spectrometry.



R_1 = CH_3; R_2 = CH_2-CH_3 (**24, Iva**)
R_1 = CH_2-CH_3; R_2 = CH_2-CH_3 (**25, Deg**)
R_1 = $(CH_2)_2$-CH_3; R_2 = $(CH_2)_2$-CH_3 (**26, Dpg**)

Scheme 1. Synthesis of the amino acids **Iva**; **Deg**; **Dpg**. Reagents and conditions: (a) $(Bu)_4NCN$, $(NH_4)_2CO_3$, 40 h reflux; (b) $Ba(OH)_2$, 40 h 140 °C.

observed with these Aib analogues may derive from a negative effect on the alpha helical conformation of [Aib^7]N/OFQ-NH_2.[16] Therefore, the steric hindrance of the amino acid side chain in position 7, which was previously suggested to be important for optimal NOP receptor interaction,[16] is probably responsible for the moderate decrease in potency obtained with these analogues. Indeed, among compounds **1**–**11** the larger is the side chain in position 7 the lower the potency of the corresponding N/OFQ-NH_2 derivative. The only exception to this rule is compound **1** in which Ac_3c, a cyclic analogue of Aib with the same number of carbon atom in the side chain, produced a more than 10-fold reduction in potency. This finding requires further comments. The τ bond angle (the Nα–Cα–CO bond angle) of Aib and Ac_3c is ≈110° and ≈117°, respectively.[29] The larger τ bond angle of Ac_3c could induce a distortion

Table 2. Effects of N/OFQ-NH_2 and N/OFQ-NH_2 analogues in the electrically stimulated mouse vas deferens

No.	Abbreviated names	AGONIST	
		pEC_{50} ($CL_{95\%}$)	E_{max} (%)
	N/OFQ-NH_2	7.80 (7.74–7.86)	93 ± 2
	[Aib^7]N/OFQ-NH_2	8.35 (8.24–8.46)	96 ± 3
	[Aib^{11}]N/OFQ-NH_2	8.05 (7.88–8.22)	91 ± 5
1	[Ac_3c^7]N/OFQ-NH_2	7.08 (6.82–7.34)	98 ± 1
2	[Ac_5c^7]N/OFQ-NH_2	7.60 (7.40–7.80)	98 ± 1
3	[Ac_6c^7]N/OFQ-NH_2	7.20 (6.86–7.54)	87 ± 1
4	[(αMe)D/L-Val^7]N/OFQ-NH_2(1)	7.26 (7.00–7.52)	88 ± 1
5	[(αMe)D/L-Val^7]N/OFQ-NH_2(2)	7.56 (7.34–7.78)	95 ± 1
6	[(αMe)D/L-Leu^7]N/OFQ-NH_2(1)	7.33 (7.04–7.62)	84 ± 1
7	[(αMe)D/L-Leu^7]N/OFQ-NH_2(2)	7.12 (7.02–7.22)	95 ± 2
8	[Iva^7]N/OFQ-NH_2(1)	7.83 (7.74–7.92)	91 ± 4
9	[Iva^7]N/OFQ-NH_2(2)	7.62 (7.32–7.92)	88 ± 3
10	[Deg^7]N/OFQ-NH_2	7.91 (7.53–8.27)	89 ± 2
11	[Dpg^7]N/OFQ-NH_2	7.90 (7.71–8.11)	91 ± 4
12	[Ac_3c^{11}]N/OFQ-NH_2	7.78 (7.62–7.94)	91 ± 4
13	[Ac_5c^{11}]N/OFQ-NH_2	8.08 (7.93–8.23)	89 ± 4
14	[Ac_6c^{11}]N/OFQ-NH_2	7.79 (7.53–8.05)	89 ± 4
15	[(αMe)D/L-Val^{11}]N/OFQ-NH_2 (1)	7.71 (7.37–8.05)	93 ± 2
16	[(αMe)D/L-Val^{11}]N/OFQ-NH_2 (2)	7.83 (7.67–7.99)	86 ± 4
17	[(αMe)D/L-Leu^{11}]N/OFQ-NH_2	7.87 (7.67–8.07)	91 ± 4
18	[D/L-Iva^{11}]N/OFQ-NH_2	8.12 (7.78–8.46)	90 ± 4
19	[Deg^{11}]N/OFQ-NH_2	7.75 (7.43–7.89)	87 ± 4
20	[Dpg^{11}]N/OFQ-NH_2	7.53 (7.17–8.04)	86 ± 3

Data are means ± SEM of at least 4 separate experiments.

of the C-terminal helix and/or could limit the formation of the beta bend centred on Gly^6-Ala^7 sequence which may be important for N/OFQ biological activity.[30]

Independent of the effects of side chain size, the negligible differences in potency between the three couples of diastereomers (compounds **4**–**5**, **6**–**7** and **8**–**9**) suggest that the inversion of chirality of the amino acid residue in position 7 of the peptide sequence does not affect the screw sense of the helix that remains a right-handed helix as consistently reported for both N/OFQ[18] and its Aib related analogues.[16]

The same Cα,α-disubstituted amino acids were employed to replace Aib^{11} in the N/OFQ-NH_2 sequence. Results summarized in Table 2 indicate that position 11 is less sensitive than position 7 to these chemical modifications. Indeed, replacement with Ac_5c (compound **13**) or Iva (compound **18**) generated analogues equipotent to [Aib^{11}]N/OFQ-NH_2, while the insertion of all the other Cα,α-disubstituted amino acids (compounds **12**, **14**–**17**, and **19**–**20**) produced a moderate (about 3-fold) decrease in potency, making these peptides equipotent to N/OFQ-NH_2.

In conclusion, despite the large battery of Cα,α-disubstituted amino acids employed for substituting Aib in position 7 and 11, the most potent N/OFQ analogue of this series was [Aib^7]N/OFQ-NH_2. On this basis, we include/add this chemical modification to the most useful NOP receptor ligands that we previously identified[12] thus generating compound **21** [Nphe^{1}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2 (coded as UFP-111), compound **22** [(pF)Phe^{4}Aib^{7}Arg^{14} Lys^{15}]N/OFQ-NH_2 (UFP-112) and compound **23** [Phe$^1\Psi$(CH_2–NH)Gly^2(pF)Phe^{4}Aib^7-Arg^{14}Lys^{15}]N/OFQ-NH_2 (UFP-113). These novel NOP receptor ligands were characterized pharmacologically in a series of in vitro assays including receptor binding and functional [^{35}S]GTPγS binding in membranes from CHO cells stably expressing the human NOP receptor and in bioassay experiments performed in the electrically stimulated mouse and rat vas deferens and guinea pig ileum tissues.

The ability of UFP-111, UFP-112 and UFP-113 to bind to NOP and classical opioid receptors (MOP, DOP and KOP) was evaluated using membranes of CHO cells expressing the human recombinant proteins. In

Table 3. Receptor binding profile of the novel NOP receptor ligands

	Membranes of CHO cells expressing the human recombinant receptor			
	NOP	MOP	DOP	KOP
Control ligand	N/OFQ	DAMGO	Naltrindole	Nor-BNI
	9.50	8.43	9.97	9.90
UFP-111	9.75	<5	<5	6.17
UFP-112	10.55	7.13	6.37	8.36
UFP-113	10.26	6.45	5.69	7.55

Data are expressed as pK_i values obtained from 3 separate experiments.

Table 4. Effects of novel NOP receptor ligands in various functional assays

	GTPγS binding assay						Electrically stimulated isolated tissues					
	CHO_{hNOP} cell membranes			Mouse vas deferens			Guinea pig ileum			Rat vas deferens		
	Agonist		Antagonist	Agonist		Antagonist	Agonist		Antagonist	Agonist		Antagonist
	pEC_{50}($CL_{95\%}$)	E_{max}	pA_2($CL_{95\%}$)	pEC_{50}($CL_{95\%}$)	E_{max}	pA_2($CL_{95\%}$)	pEC_{50}($CL_{95\%}$)	E_{max}	pA_2($CL_{95\%}$)	pEC_{50}($CL_{95\%}$)	E_{max}	pA_2($CL_{95\%}$)
N/OFQ	9.04 (8.80–9.28)	100%	ND	7.45 (7.31–7.59)	87 ± 2%	ND	8.05 (7.89–8.21)	45 ± 3%	ND	6.83 (6.62–7.04)	79% ± 3%	ND
UFP-111	Inactive		8.68 (8.45–8.91)	Inactive		7.46 (7.31–7.61)	Inactive		7.33 (7.19–7.47)	Inactive		7.06 (6.84–7.28)
UFP-112	10.55 (10.30–10.80)	103 ± 7%	ND	9.24 (8.97–9.51)	84 ± 6%	ND	9.17 (8.88–9.46)	32 ± 3%	ND	8.34 (7.87–8.81)	90 ± 2%	ND
UFP-113	9.72 (9.51–9.93)	79 ± 3%	ND	Variable agonist effects		9.10 (8.95–9.30)	Variable agonist effects		9.50 (8.20–9.80)	Variable agonist effects		9.22 (8.72–9.72)

Data are means ± SEM of at least 4 separate experiments.
ND: not determined because of the agonist activity of the ligand.

CHO_{hNOP} membranes, the three peptides produced concentration-dependent inhibition of [^{3}H]N/OFQ binding with very high pK_i values (9.75–10.55), displaying an affinity for the NOP receptor higher than that of the natural ligand N/OFQ (pK_i = 9.50) (Table 3). UFP-111, UFP-112 and UFP-113 displayed high to excellent selectivity over classical opioid receptors corresponding to >100-fold for UFP-112, >300-fold for UFP-113, and >1000-fold for UFP-111 (Table 3). Standard ligands for classical opioid receptor displayed affinity values consistent with those reported in the literature.[31]

In CHO_{hNOP} cell membranes N/OFQ stimulated GTPγ[^{35}S] binding in a concentration dependent manner with a pEC_{50} of 9.04 and maximal effect corresponding to approx 8-fold over basal values (Table 4). UFP-112 and UFP-113 mimicked the stimulatory effect of N/OFQ but were 32- and 5-fold more potent, respectively. In addition, the maximal effects elicited by UFP-112 were similar to those induced by N/OFQ, while those produced by UFP-113 corresponded to 79% of those of the natural peptide: thus UFP-113 behaved in this assay as a high efficacy partial agonist (Table 4). UFP-111 up to 1 μM did not elicit any stimulation of GTPγ[^{35}S] binding in CHO_{hNOP} cell membranes, however it produced a concentration dependent and parallel shift of the concentration response curve to N/OFQ without modifying the maximal effects induced by the agonist (Fig. 1, top panels). Schild analysis of these data was compatible with a competitive type of antagonism and a pA_2 value of 8.68 was derived from these experiments.

In conclusion, experiments performed using cells expressing recombinant receptors demonstrated that UFP-111, UFP-112 and UFP-113 are highly potent and selective ligands for the NOP receptor encompassing full (UFP-112) and partial (UFP-113) agonist as well as pure antagonist (UFP-111) behaviour.

In the electrically stimulated mouse vas deferens UFP-112 concentration dependently inhibited electrically induced twitches eliciting maximal effects similar to N/OFQ but was 79-fold more potent (Table 4). It is worthy of mention that the kinetics of the inhibitory effects evoked by N/OFQ and UFP-112 were very different. The inhibitory effects induced by N/OFQ occurred immediately after adding the peptide to the bath and were rapidly (≈3 min) reversible after washing of the tissue. In contrast, UFP-112 induced slower inhibitory effects which reached a plateau only after ≈10 min; moreover, tissues treated with UFP-112 did not fully recover to the control twitch even after washing the tissue for more than 2 h.

UFP-113 produced variable agonist effects in this preparation with 10 out of 13 tissues sensitive to its action, which was always less than 50% inhibition. However, when preincubated for 15 min at 10 nM, UFP-113 consistently produced a concentration dependent and paral-

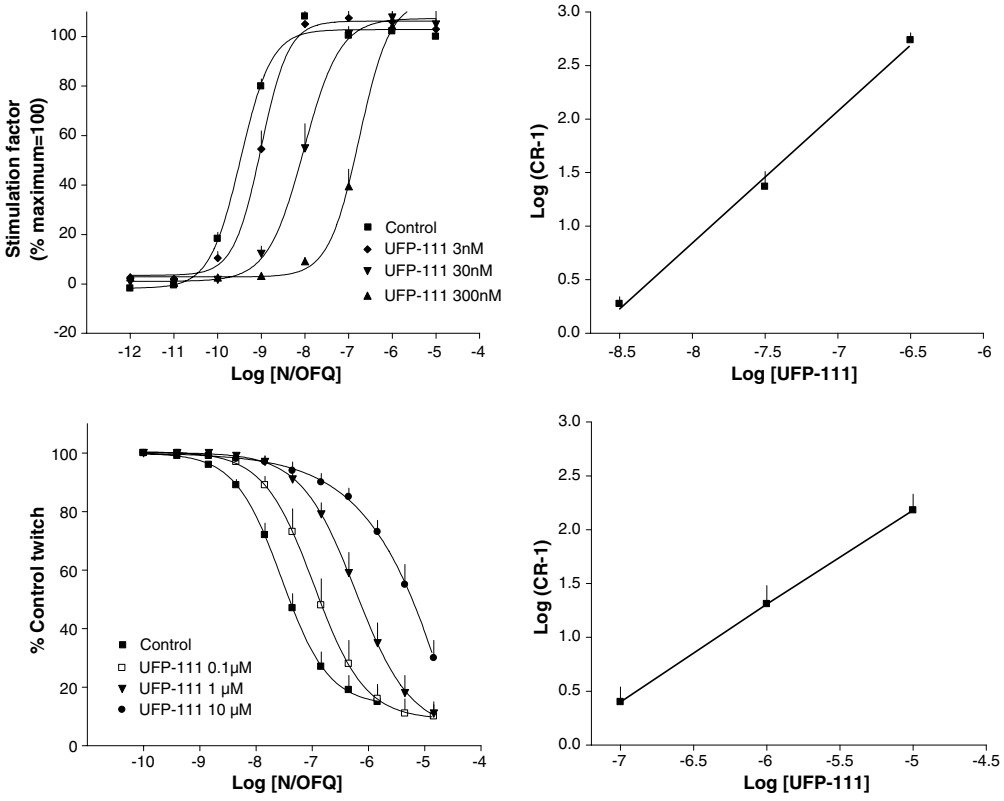


Figure 1. Concentration response curves to N/OFQ in the absence and presence of increasing concentrations of UFP-111 on GTPγS binding in CHO_{hNOP} cell membranes (top panels) and on electrically induced twitching of the mouse vas deferens (bottom panels). The corresponding Schild regression plots are depicted in the right panels. Data are means ± SEM of 4–6 experiments.

lel rightward shift of the concentration response curve to N/OFQ with no modifications of the maximal agonist effects: a pA_2 value of 9.24 was calculated for UFP-113 from these experiments. UFP-111 up to 10 μM did not modify the electrically induced twitch of the mouse vas deferens but caused a concentration dependent displacement to the right of the concentration response curve to N/OFQ (Fig. 1, bottom panels). Schild analysis of the data yielded a pA_2 value of 7.46. Very similar results were obtained with the three peptides in the rat vas deferens and guinea pig ileum where UFP-112 and UFP-113 behaved as potent full and partial agonists, respectively, and UFP-111 as a pure antagonist. Thus, bioassay studies performed on isolated tissues expressing native NOP receptors from different species confirmed and extended the results obtained using the human recombinant NOP receptor demonstrating that UFP-112, UFP-113, and UFP-111 act as potent NOP receptor full agonist, partial agonist and pure antagonist, respectively.

These conclusions are corroborated by the recent findings by Chang et al.[32] These authors synthesised and evaluated in receptor binding (rat brain membranes) and bioassay (mouse vas deferens) experiments a series of N/OFQ related peptides very similar to those described in this paper with similar findings: for instance the peptide [(pF)Phe^{4}Aib^{7}Aib^{11}Arg^{14}Lys^{15}]N/OFQ-NH_2 displayed an affinity value of 10.78 and an agonist potency in the mouse vas deferens assay of 9.37 (present results UFP-112 pK_i 10.55, pEC_{50} 9.24), and the peptide [Nphe^{1}Aib^{7}Aib^{11}Arg^{14}Lys^{15}]N/OFQ-NH_2 an affinity value of 9.58 and an antagonist potency in the mouse vas deferens assay of 8.01 (present results UFP-111 pK_i 9.75, pA_2 7.46). This confirms[12] that several different chemical modifications can be combined in the N/OFQ sequence for increasing peptide affinity/potency and for modulating peptide efficacy. The pharmacological features of novel peptide NOP ligands generated via such an approach appear to be highly consistent across different assays (receptor binding and functional biochemical studies on the recombinant receptor, receptor binding and bioassay on tissues expressing the native receptor), different laboratories (our laboratory and that of Prof. Rui Wang) and different pharmacological entities (full agonists and pure antagonists). The present results extend these findings to NOP receptor partial agonists (i.e., UFP-113), a pharmacological species highly interesting for physiopathological studies and possibly therapeutic applications. In fact, it has been demonstrated that NOP receptor partial agonists are able to mimic some N/OFQ effects that can be exploited for therapeutic purposes (i.e., inhibition of micturition reflex,[33] induction of aquaresis[34]) without producing concurrent side effects at cardiovascular level such as bradycardia and hypotension.

The fact that superimposable results were obtained with our peptides (modified only in position 7) and with those of the Chinese group (substituted with Aib both in position 7 and 11) is in line with findings reported in previous studies performed with the natural N/OFQ sequence;[15,16] this evidence strongly suggests that a single substitution with Aib (either in position 7 or 11) is sufficient to impart helicity to the C-terminus of the molecule producing the subsequent increase in potency. In addition the present findings demonstrate that this mechanism operates independently from the ligand efficacy and indeed a similar increase in potency was obtained with the full agonist UFP-112, the partial agonist UFP-113 and the pure antagonist UFP-111.

The actual impact of the Aib^7 substitution on the pharmacological properties of the present series of NOP receptor ligands can be derived by comparing the present data with those reported in Guerrini et al.[12] where the same peptides with Ala in position 7 were evaluated in the same panel of assays (receptor binding and GTPγ[^{35}S] binding in CHO_{hNOP} cell membranes, bioassay in the electrically stimulated mouse vas deferens and guinea pig ileum tissues). From such comparison it emerges that the in vitro biological activities of the compounds belonging to the two groups (with and without Aib^7) are very similar both in terms of pharmacological behaviour (full and partial agonist, pure antagonist) and values of affinity/potency. Thus, based on in vitro results it should be concluded that the Aib^7 substitution is not able to further increase peptide affinity/potency, at least in the present series of ligands. However, this apparently negative finding is not completely unexpected because, when the affinities/potencies of peptide analogues reach levels 30 or more fold higher than those of the corresponding reference compound (i.e., N/OFQ for UFP-112, [Nphe^1]N/OFQ for UFP-111 and [Phe$^1\Psi$(CH_2–NH)Gly^2]N/OFQ for UFP-113) it might be very difficult to further improve affinity/potency due to ceiling effects. Nevertheless this does not exclude that the in vivo features of the two series (Ala^7 and Aib^7 derivatives) of compounds might be different.

Interestingly enough, UFP-112 has been recently evaluated in vivo in mice where it mimicked the supraspinal pronociceptive, orexigenic and hypolocomotor actions and spinal pronociceptive effects of N/OFQ.[35] However, in these assays UFP-112 was 100-fold more potent than N/OFQ and elicited longer lasting effects. In addition in vivo NOP receptor knockout mice experiments demonstrated the exclusive involvement of the NOP protein in the actions elicited by both N/OFQ and UFP-112[35] thus confirming the high selectivity of action of this novel peptide. Very similar results (both in terms of potency and long lasting action) were obtained by the Wang group investigating the supraspinal pronociceptive action and the hypotensive effect of [(pF)Phe^{4}Aib^{7}Aib^{11} Arg^{14}Lys^{15}]N/OFQ-NH_2.[36]

As far as the actual impact of the Aib^7 substitution on the ligand in vivo pharmacological properties is concerned, it is worthy of note that the potency of UFP-112 given icv in the mouse tail withdrawal assay[35] is approx. 10-fold higher than that of corresponding peptide without Aib^7 (UFP-102,[13]). Thus, replacement of Ala by Aib in position 7 seems to be particularly important for the in vivo potency of NOP peptide ligands: this may derive from the Aib^{7}induced alpha

helix conformation which is known to reduce peptide susceptibility to peptidase action.[37–39]

3. Conclusion

In conclusion, the present study demonstrated that among a large collection of Cα,α-disubstituted amino acids known to be compatible with alpha helical structures, employed for substituting Ala^7 and Ala^{11} in the N/OFQ sequence, the most interesting results in terms of biological activity were obtained using Aib in position 7. This substitution was then combined with other chemical modifications and series of novel highly potent peptide ligands for the NOP receptor encompassing full (UFP-112) and partial (UFP-113) agonist, and pure antagonist (UFP-111) activities were generated. These peptides represent the most potent and NOP selective peptide ligands currently available.

4. Experimental

4.1. Materials

Amino acids, protected amino acids and chemicals were purchased from Bachem, Novabiochem, Fluka or Chem-Impex International.The resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxy-phenoxy)-valeric acid]-polyethyleneglycol/polystyrene support (Fmoc-PAL-PEG-PS) was from Millipore (Waltham, MA). All other reagents were from Sigma Chemical Co. (Poole, UK) or E. Merck (Darmstadt, Germany) and were of the highest purity available. Melting points (uncorrected) were measured with a Buchi–Tottoli apparatus, and ^1H, ^{13}C and NMR spectra were recorded on a VARIAN 400 MHz instrument unless otherwise noted. Chemical shifts are given in ppm (δ) relative to TMS and coupling constants are in Hz. Mass analyses were performed on a ESI-Micromass ZMD 2000. TLC was performed on precoated plates of silica gel F254, while flash chromatography was carried out on a silica gel Merck, 230–400 Mesh.

4.2. General procedure for the hydantoin preparation

To a stirred solution of ketone (20 mmol) dissolved in a 1:1 mixture of H_2O/Et-OH (100 mL), $(NH_4)_2CO_3$ (2 mmol) and tetrabutylammonium cyanide (12.3 mmol) were added. The reaction was refluxed for 40 h. After the evaporation of the solvent the crude material was purified by flash chromatography using CH_2Cl_2/MeOH; 9.5:0.5 as mobile phase.

4.2.1. 5-Ethyl-5methyl-imidazolidin-2,4-dione (24). Yield 50%; mp 141–143 °C; ^1H NMR ($CDCl_3$): δ 8.62 (s, 1H, NH), 6.18 (s, 1H, NH), 1.92 (m, 2H), 1.44 (s, 3H), 1.04 (m, 3H); ^{13}C NMR ($CDCl_3$): 177.7, 157.0, 64.4, 31.0, 23.7, 8.1; MS: $[M+H]^+$ calcd 143, found 143.1.

4.2.2. 5,5-Diethyl-imidazolidin-2,4-dione (25). Yield 25%; mp 161–163 °C; ^1H NMR ($CDCl_3$): δ 9.02 (s, 1H, NH), 6.51 (s, 1H, NH), 1.90 (m, 4H, $2CH_2$), 0.99 (m, 6H, $2CH_3$); ^{13}C NMR ($CDCl_3$): 177.5, 158.2, 68.5, 29.8 (2 carbon atoms), 7.9 (2 carbon atoms); $[M+H]^+$ calcd 157, found 157.2.

4.2.3. 5,5-Dipropyl-imidazolidin-2,4-dione (26). Yield 25%; mp 188–191 °C; ^1H NMR ($CDCl_3$): δ 9.97 (s, 1H, NH), 6.66 (s, 1H, NH), 1.75 (m, 4H, $2CH_2$), 1,35 (m 4H $2CH_2$), 0.99 (m, 6H, $2CH_3$); ^{13}C NMR ($CDCl_3$): 178.3, 160.2, 64.9, 37.7 (2 carbon atoms), 14.8 (2 carbon atoms), 13.6 (2 carbon atoms); $[M+H]^+$ calcd 185, found 185.3.

4.3. General procedure for the hydantoin hydrolysis

To a suspension of hydantoin (2.53 mmol) in water (10 mL), barium hydroxide octahydrate (12.68 mmol) was added and the mixture stirred at 140 °C for 48 h. After this time the mixture was acidified with sulfuric acid (6 N) to pH 4 and then filtered on gooch funnel. The solution was evaporated under reduced pressure and the residue lyophilized.

4.3.1. 2-Methyl,2-amino-butanoic acid (27). Yield 98%; mp >300 °C; ^1H NMR (DMSO): δ 1.78 (q, 2H, CH_2), 1.33 (s, 3H, CH_3), 0.96 (t, 3H, CH_3); ^{13}C NMR (DMSO): 179.5, 64.4, 32.6, 23.6, 5.8; $[M+H]^+$ calcd 117, found 117.8.

4.3.2. 2-Ethyl,2-amino-butanoic acid (28). Yield 98%; mp >300 °C; ^1H NMR (DMSO): δ 1.78 (q, 4H, CH_2), 0.92 (t, 6H, CH_3); ^{13}C NMR (DMSO): 181.0, 67.4, 30.1 (2 carbon atoms), 6.2 (2 carbon atoms); $[M+H]^+$ calcd 132, found 131.8.

4.3.3. 2-Propyl,2-amino-pentanoic acid (29). Yield 98%; mp >300 °C; ^1H NMR (DMSO): δ 1.74 (t, 4H, CH_2), 1.33 (m, 4H, CH_2), 0.96 (t, 6H, CH_3); ^{13}C NMR (DMSO): 182.0, 62.4, 39.9 (2 carbon atoms), 15.2 (2 carbon atoms), 13.1 (2 carbon atoms); $[M+H]^+$ calcd 160, found 160.2.

4.4. General procedure for Fmoc-amino acid protection

To a stirred solution of the amino acid (4.53 mmol) in water (10 mL), Na_2CO_3 (9.06 mmol) and, dropwise, Fmoc–Cl (5.44 mmol) in dioxane (10 mL) were added at 0 °C. After 8 h at room temperature the reaction was quenched with HCl 1 N (50 mL) and the mixture extracted with ethyl acetate (3× 20 mL). The organic layer was anhydrified with sodium sulfate anhydrous and evaporated under reduced pressure. The crude residue was purified by flash chromatography using DCM/MeOH 9.5:0.5 as eluant.

4.4.1. Fmoc-Iva. Yield 32%; mp 105–107 °C; ^1H NMR ($CDCl_3$): δ 7.68–7.17 (m, 8H), 4.25 (d, 2H, CH_2–O, $J = 6.8$), 4.07 (t, 1H, $J = 6.8$), 1.79 (m, 5H), 0.65 (t, 3H, $J = 6.9$); ^{13}C NMR ($CDCl_3$): 178.7, 155.7, 143.9 (2 carbon atoms), 141.3 (2 carbon atoms), 127.7 (2 carbon atoms), 127.0 (2 carbon atoms), 125.1 (2 carbon atoms), 119.9 (2 carbon atoms), 65.9, 60.2, 47.2, 20.2, 15.3, 8.5; $[M+H]^+$ calcd 340, found 340.2.

4.4.2. Fmoc-Deg. Yield 40%; mp 103–105 °C; ^1H NMR ($CDCl_3$): δ 7.77–7.29 (m, 8H), 4.40 (d, 2H, CH_2–O,

J = 6.8), 4.21 (t, 1H, J = 6.8), 2.32 (q, 2H, J = 7.2), 1.86 (8H), 4.40 (d, 2H, CH_2–O, J = 6.8), 4.21 (t, 1H, J = 6.8), 2.32 (q, 2H, J = 7.2), 1.86 (q, 2H, J = 7.2), 0.83 (t, 6H, 2CH_3, J = 6.4); ^{13}C NMR ($CDCl_3$): 180.0, 156.0, 142.7 (2 carbon atoms), 141.4 (2 carbon atoms), 127.7 (2 carbon atoms), 127.1 (2 carbon atoms), 125.1 (2 carbon atoms), 120.1 (2 carbon atoms), 66.3, 63.1, 47.3, 28.3 (2 carbon atoms), 8.4 (2 carbon atoms); $[M+H]^+$ calcd 354, found 354.4.

4.4.3. Fmoc-Dpg. Yield 87%; mp 102–105 °C; ^{1}H NMR ($CDCl_3$): δ 7.69–7.15 (m, 8H), 4.28 (d, 2H, CH_2–O, J = 6.8), 4.09 (t, 1H, J = 6.8), 2.16 (m, 14H); ^{13}C NMR ($CDCl_3$): 180.2, 155.7, 144.1 (2 carbon atoms), 141.5 (2 carbon atoms), 127.9 (2 carbon atoms), 127.3 (2 carbon atoms), 125.2 (2 carbon atoms), 120.2 (2 carbon atoms), 64.3, 58.0, 47.5, 38.0 (2 carbon atoms), 17.6 (2 carbon atoms), 14.3 (2 carbon atoms); $[M+H]^+$ calcd 381, found 382.3.

4.5. General procedures for the solid phase synthesis

As an illustrative example the synthesis of [Ac$_3$c^7]N/OFQ-NH$_2$ (**1**) is described. Fmoc-PAL-PEG-PS resin (0.21 mmol/g, 0.5 g) was treated with 20% piperidine/DMF and linked with Fmoc-Gln(Trt)-OH by using [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) as the coupling reagent. The following N^{α}-Fmoc amino acids were sequentially coupled to the growing peptide chain: Fmoc-Asn(Trt)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ala-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ac$_3$c-OH, Fmoc-Gly-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Phe-OH. All the N^{α}-Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using HATU (4 equiv) in DMF in the presence of an equimolar concentration of 4-methylmorpholine, and the coupling reaction time was 1 h. 20% Piperidine/DMF was used to remove the Fmoc group at every step. The peptide resin was washed with methanol and dried in vacuo to yield the protected [Ac$_3$c^7]N/OFQ-NH$_2$-Resin. The other peptides **2**–**23** were synthesised in a similar manner. The protected peptide-resin was treated with reagent K[40] (TFA/H_2O/phenol/ethanedithiol/thioanisole 82.5:5:5:2.5:5; v/v; 10 mL/0.2 g of resin) for 1 h at room temperature. After filtration of the exhausted resin, the solvent was concentrated in vacuo and the residue triturated with ether. The crude peptide was purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

4.6. Peptide purification and analytical determinations

Crude peptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0% to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Analytical purity and retention time (t_R) of the peptides were determined using two different HPLC conditions.

Retention time I was obtained using a Vydac C_{18} column (4.6 × 100 mm, 2 μm particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 1 mL/min using a linear gradient from 0% to 30% B over 25 min. Retention time II was obtained using a TSKgel super ODS C_{18} column (4.6 × 150 mm, 5 μm particle size) with solvent A: 35 mM NaH_2PO_4 (pH 2.1) and solvent B: 59 mM NaH_2PO_4 (pH 2.1)-acetonitrile (60:40, v/v). The column was perfused at a flow rate of 1 mL/min with a linear gradient from 10% to 65% B over 25 min. The amino acids (αMe)-Val, (αMe)-Leu and Iva were used, for the synthesis of the corresponding N/OFQ analogues, as racemic mixture; (1) and (2) in Tables 1 and 2 indicate the diastereomeric N/OFQ analogue with the earlier and the later elution times, respectively. In the case of compounds **17** and **18** the preparative HPLC separation of the two diastereomers was not base peak resolved and consequently these compounds were purified and tested as diastereomeric mixture. For these compounds the retention time reported in Table 1 is the mean of the retention time of the two peaks.

All analogues showed >95% purity when monitored at 220 nm. Molecular weights of compounds were determined by a mass spectrometer ESI Micromass ZMD-2000, values are expressed as MH^+. The analytical properties of N/OFQ analogues are reported in Table 1.

4.7. Cell cultures

CHO_{hNOP} cells were maintained in Dulbecco's MEM/Hams F12 (1:1) supplemented with 5% FCS, penicillin (100 IU/mL), streptomycin (100 μg/ml) and fungizone (2.5 μg/mL). Media for stock (non experimental) cultures were further supplemented with hygromycin B (200 μg/mL) and geneticin (G418) (200 μg/mL). CHO cells expressing the human recombinant KOP (kappa), DOP (delta) and MOP (mu) opioid receptors were cultured in Hams F12 with 10% foetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/mL). Stock cultures additionally contained geneticin (200 μg/mL) for maintenence of the receptor plasmid. Cell cultures were maintained at 37 °C in 5% CO_2/humidified air and used for experimentation when confluent (3–4 days). For GTPγ[^{35}S] and [*leucyl*-^{3}H]N/OFQ competition binding studies membrane fragments obtained through homogenisation of cell suspensions followed by centrifugation (20,000*g*, for 10 min at 4 °C) in buffer consisting of either Tris (50 mM), with or without $MgSO_4$ (5 mM) (competition binding for NOP or classical opioid, respectively) or Tris (50 mM) EGTA (0.2 mM) (GTPγ[^{35}S] binding) were used. Membrane fragments were further homogenised and centrifuged for a total of three cycles.

4.8. Competition binding assay

Ten micrograms of CHO_{hNOP} membrane protein was incubated in 0.5 mL of homogenisation buffer containing 0.5% BSA, 10 μM of a cocktail of peptidase inhibitors (captopril, amastatin, bestatin and phosphoramidon), ~0.2 nM [^{3}H]N/OFQ and increasing concentrations (10^{-12}–10^{-5} M) of the non-radiolabelled peptides under study. Non-specific binding was defined in the presence of 1 μM N/OFQ. For opioid receptor selectivity studies, the ability of increasing concentrations of peptides to displace the binding of [^{3}H]diprenorphine was measured at human recombinant classical opioid receptors. Between 30 and 45 μg of $CHO_{hKOP/DOP/MOP}$ membranes was incubated in homogenisation buffer containing 10 μM peptidase inhibitors (as above) and ~0.4 nM [^{3}H]diprenorphine. Non-specific binding was defined in the presence of 10 μM naloxone. All competition binding studies were incubated for 1 h at room temperature and bound and free radioactivity was separated by vacuum filtration using a Brandel cell harvester using Whatman GF/B filters soaked in polyethylenimine (0.5%) to reduce non-specific binding.

4.9. GTPγ[^{35}S] binding assay

Experimentation was performed essentially as described by Berger et al.[41] Freshly, prepared CHO_{hNOP} membranes (20 μg) were incubated in 0.5 mL volumes of buffer consisting Tris (50 mM), EGTA (0.2 mM), GDP (100 μM), bacitracin (0.15 mM), BSA (1 mg/mL), peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon; 10 μM), GTPγ[^{35}S] (~150 pM) and peptides in the concentration range of 10^{-12}–10^{-5} M in various combinations. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. Assays were incubated for 1 h at 30 °C with gentle shaking and bound and free radiolabel were separated by vacuum filtration onto Whatman GF/B filters. Polyethylenimine was not used. In all cases radioactivity was determined following filter extraction (8 h, Optiphase Safe, Wallac) using liquid scintillation spectroscopy.

4.10. Bioassay on electrically stimulated tissues

Tissues were taken from male Swiss mice (30–35 g), Sprague–Dawley rats (300–350 g) and albino guinea pigs (300–350 g). The mouse and rat vas deferens and the guinea pig ileum tissues were prepared as previously described.[42] Tissues were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 m s duration and 0.05 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006) and recorded with the PC based acquisition system PowerLab 4/25 (model ML845, ADInstruments, Australia). After an equilibration period of about 60 min, the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration-response curves to N/OFQ or to the peptides under study were performed (0.5 log-unit step). In some experiments the antagonistic properties of UFP-113 and UFP-111 were assessed by comparing the concentration-response curves to N/OFQ in their absence and presence (15 min pre-incubation time).

4.11. Data analysis and terminology

All data are expressed as means ± standard error of the mean (SEM), for potency values confidence limits 95% were indicated. The number of separate experiments is reported for each series of data. Data have been analyzed statistically with Student's *t*-test or one way ANOVA followed by the Dunnett test, as specified in table and figure legends; p values less than 0.05 were considered statistically significant. Concentration of ligands producing 50% inhibition of specific binding (IC_{50}) was corrected for the competing mass of radioligand using the Cheng and Prusoff equation to yield K_i values. Curve fitting was performed using PRISM 4.0 (GraphPad Software Inc., San Diego, USA). Agonist potencies were expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given tissue/preparation. pA_2 values were calculated using the Gaddum Schild equation $pA_2 = -\log((CR - 1)/[\text{Antagonist}])$, assuming a slope equal to unity. For the effects of UFP-111 in the GTPγ[^{35}S] binding and mouse vas deferens assay the classical Schild analysis was performed by testing the antagonist at three different concentrations over a 2 log unit range.

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References and notes

1. Meunier, J. C.; Mollereau, C.; Toll, L.; Suaudeau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monserrat, B.; Mazarguil, H.; Vassart, G.; Parmentier, M.; Costentin, J. *Nature* **1995**, *377*, 532.
2. Reinscheid, R. K.; Nothacker, H. P.; Bourson, A.; Ardati, A.; Henningsen, R. A.; Bunzow, J. R.; Grandy, D. K.; Langen, H.; Monsma, F. J., Jr.; Civelli, O. *Science* **1995**, *270*, 792.
3. Calo, G.; Guerrini, R.; Rizzi, A.; Salvadori, S.; Regoli, D. *Br. J. Pharmacol.* **2000**, *129*, 1261.
4. Cox, B. M.; Chavkin, C.; Christie, M. J.; Civelli, O.; Evans, C.; Hamon, M. D.; Hoellt, V.; Kieffer, B.; Kitchen, I.; McKnight, A. T.; Meunier, J. C.; Portoghese, P. S. Opioid receptors. In *The IUPHAR Compendium of Receptor Characterization and Classification*, 2nd ed.; IUPHAR Media Ltd: London, 2000; pp 321–333.
5. Bignan, G. C.; Connolly, P. J.; Middleton, S. A. *Expert Opin. Ther. Patents* **2005**, *15*, 357.
6. Zaveri, N. *Life Sci.* **2003**, *73*, 663.
7. Guerrini, R.; Calo, G.; Bigoni, R.; Rizzi, D.; Rizzi, A.; Zucchini, M.; Varani, K.; Hashiba, E.; Lambert, D. G.; Toth, G.; Borea, P. A.; Salvadori, S.; Regoli, D. *J. Med. Chem.* **2001**, *44*, 3956.

8. Okada, K.; Sujaku, T.; Chuman, Y.; Nakashima, R.; Nose, T.; Costa, T.; Yamada, Y.; Yokoyama, M.; Nagahisa, A.; Shimohigashi, Y. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 493.
9. Guerrini, R.; Calo, G.; Rizzi, A.; Bigoni, R.; Bianchi, C.; Salvadori, S.; Regoli, D. *Br. J. Pharmacol.* **1998**, *123*, 163.
10. Calo, G.; Guerrini, R.; Bigoni, R.; Rizzi, A.; Bianchi, C.; Regoli, D.; Salvadori, S. *J. Med. Chem.* **1998**, *41*, 3360.
11. Guerrini, R.; Calo, G.; Bigoni, R.; Rizzi, A.; Varani, K.; Toth, G.; Gessi, S.; Hashiba, E.; Hashimoto, Y.; Lambert, D. G.; Borea, P. A.; Tomatis, R.; Salvadori, S.; Regoli, D. *J. Med. Chem.* **2000**, *43*, 2805.
12. Guerrini, R.; Calo, G.; Lambert, D. G.; Carra, G.; Arduin, M.; Barnes, T. A.; McDonald, J.; Rizzi, D.; Trapella, C.; Marzola, E.; Rowbotham, D. J.; Regoli, D.; Salvadori, S. *J. Med. Chem.* **2005**, *48*, 1421.
13. Carra, G.; Rizzi, A.; Guerrini, R.; Barnes, T. A.; McDonald, J.; Hebbes, C. P.; Mela, F.; Kenigs, V. A.; Marzola, G.; Rizzi, D.; Gavioli, E.; Zucchini, S.; Regoli, D.; Morari, M.; Salvadori, S.; Rowbotham, D. J.; Lambert, D. G.; Kapusta, D. R.; Calo, G. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 1114.
14. Calo, G.; Guerrini, R.; Rizzi, A.; Salvadori, S.; Burmeister, M.; Kapusta, D. R.; Lambert, D. G.; Regoli, D. *CNS Drug Rev.* **2005**, *11*, 97.
15. Zhang, C.; Miller, W.; Valenzano, K. J.; Kyle, D. J. *J. Med. Chem.* **2002**, *45*, 5280.
16. Tancredi, T.; Carra, G.; Guerrini, R.; Arduin, M.; Calo, G.; Regoli, D.; Salvadori, S.; Temussi, P. A. *Chemistry* **2005**, *11*, 2061.
17. Salvadori, S.; Picone, D.; Tancredi, T.; Guerrini, R.; Spadaccini, R.; Lazarus, L. H.; Regoli, D.; Temussi, P. A. *Biochem. Biophys. Res. Commun.* **1997**, *233*, 640.
18. Orsini, M. J.; Nesmelova, I.; Young, H. C.; Hargittai, B.; Beavers, M. P.; Liu, J.; Connolly, P. J.; Middleton, S. A.; Mayo, K. H. *J. Biol. Chem.* **2005**, *280*, 8134.
19. Klaudel, L.; Legowska, A.; Brzozowski, K.; Silberring, J.; Wojcik, J. *J. Pept. Sci.* **2004**, *10*, 678.
20. Topham, C. M.; Mouledous, L.; Poda, G.; Maigret, B.; Meunier, J. C. *Protein Eng.* **1998**, *11*, 1163.
21. Naydenova, E. D.; Zhivkova, V. I.; Zamfirova, R. N.; Vezenkov, L. T.; Dobrinova, Y. G.; Mateeva, P. I. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4071.
22. Calo, G.; Rizzi, A.; Bogoni, G.; Neugebauer, V.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. *Eur. J. Pharmacol.* **1996**, *311*, R3–R5.
23. Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.
24. Strecker, A. *Justus Liebigs Ann. Chem.* **1850**, *75*, 27.
25. Carpino, L. A.; Han, G. A. *J. Org. Chem.* **1972**, *37*, 3404.
26. Toniolo, C.; Crisma, M.; Formaggio, E.; Valle, G.; Cavicchioni, G.; Precigoux, G.; Aubry, A.; Kamphuis, J. *Biopolymers* **1993**, *33*, 1061.
27. Toniolo, C.; Benedetti, E. *Macromolecules* **1991**, *24*, 4004.
28. Barazza, A.; Wittelsberger, A.; Fiori, N.; Schievano, E.; Mammi, S.; Toniolo, C.; Alexander, J. M.; Rosenblatt, M.; Peggion, E.; Chorev, M. *J. Pept. Res.* **2005**, *65*, 23.
29. Crisma, M.; Bonora, G. M.; Toniolo, C.; Barone, V.; Benedetti, E.; Di Blasio, B.; Pavone, V.; Pedone, C.; Santini, A.; Fraternali, F.; Bavoso, A.; Lelj, F. *Int. J. Biol. Macromol.* **1989**, *11*, 345.
30. Amodeo, P.; Guerrini, R.; Picone, D.; Salvadori, S.; Spadaccini, R.; Tancredi, T.; Temussi, P. A. *J. Pept. Sci.* **2002**, *8*, 497.
31. Reisine, T. *Neuropharmacology* **1995**, *34*, 463.
32. Chang, M.; Peng, Y. L.; Dong, S. L.; Han, R. W.; Li, W.; Yang, D. J.; Chen, Q.; Wang, R. *Regul. Pept.* **2005**, *130*, 116.
33. Lecci, A.; Giuliani, S.; Meini, S.; Maggi, C. A. *Peptides* **2000**, *21*, 1007.
34. Kapusta, D. R.; Burmeister, M. A.; Calo, G.; Guerrini, R.; Gottlieb, H. B.; Kenigs, V. A. *J. Pharmacol. Exp. Ther.* **2005**, *314*, 643.
35. Rizzi, A.; Spagnolo, B.; Guerrini, R.; Marzola, G.; Salvadori, S.; Regoli, D.; Calo', G. *Neuroscience* 2006 Atlanta, US, 2006.
36. Peng, Y. L.; Chang, M.; Dong, S. L.; Li, W.; Han, R. W.; Fu, G. X.; Chen, Q.; Wang, R. *Regul. Pept.* **2006**, *134*, 75.
37. Shepherd, N. E.; Hoang, H. N.; Abbenante, G.; Fairlie, D. P. *J. Am. Chem. Soc.* **2005**, *127*, 2974.
38. Schafmeister, C. E.; Po, J.; Verdine, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 5891.
39. Wang, D.; Liao, W.; Arora, P. S. *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 6525.
40. King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255.
41. Berger, H.; Bigoni, R.; Albrecht, E.; Richter, R. M.; Krause, E.; Bienert, M.; Calo, G. *Peptides* **2000**, *21*, 1131.
42. Bigoni, R.; Giuliani, S.; Calo, G.; Rizzi, A.; Guerrini, R.; Salvadori, S.; Regoli, D.; Maggi, C. A. *Naunyn Schmiedebergs Arch. Pharmacol.* **1999**, *359*, 160.