N- and C-Terminal Modifications of Nociceptin/Orphanin FQ Generate Highly Potent NOP Receptor Ligands

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Previous structure−activity studies on nociceptin/orphanin FQ (N/OFQ) identified [Phe$^1\Psi$(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2 and [Nphe^1]N/OFQ(1-13)-NH_2 as a N/OFQ peptide receptor (NOP) partial agonist and pure antagonist, respectively. The addition of fluorine to the Phe^4 or the insertion of a further pair of basic amino acids Arg^{14}-Lys^{15} generate potent agonists. On the basis of these findings, we combined in the N/OFQ-NH_2 template the chemical modifications Arg^{14}-Lys^{15} and (pF)Phe^4 that increase the agonist potency with those conferring partial agonist (Phe$^1\Psi$(CH_2NH)Gly^2) or pure antagonist (Nphe^1) properties. Twelve peptides were synthesized and pharmacologically evaluated in Chinese hamster ovary cells expressing the human recombinant NOP and in electrically stimulated mouse vas deferens and guinea pig ileum assays. All peptides behaved as NOP ligands; the chemical modifications Arg^{14}-Lys^{15} and (pF)-Phe^4 increased ligand affinity/potency. Peptides with the normal Phe^1-Gly^2 peptide bond behaved as full agonists, and those with the Phe$^1\Psi$(CH_2NH)Gly^2 modification behaved as partial agonists, while those with the Nphe^1 modification behaved as partial agonists or pure antagonists depending on the presence or absence of the (pF)Phe^4 modification, respectively. The full agonist [(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2, the partial agonist [Phe$^1\Psi$(CH_2NH)-Gly^2,(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2, and the pure antagonist [Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2 represent the most potent peptide ligands for NOP.

Introduction

Nociceptin/orphanin FQ (N/OFQ)[2,3] is a neuropeptide that selectively binds and activates a G-protein-coupled receptor recently named N/OFQ peptide receptor (NOP).[4] N/OFQ and its receptor are considered "a non-opioid branch of the opioid family" of peptides and receptors.[4] This suggestion is based on the close structural and trasductional similarities that contrast with the pharmacological and functional differences between the N/OFQ-NOP and the classical opioid systems.[5,6] Understanding of the biological role of the N/OFQ-NOP system is still limited, and the identification of highly potent and selective ligands for NOP is mandatory.

The available ligands for NOP and their potential therapeutic uses have been recently reviewed.[7] They can be classified as (i) non-peptide ligands generally discovered via high-throughput screening in industrial laboratories (see the series of patents quoted by Zaveri),[7] where Ro 64-6198[8] and J-113397[9] selective NOP agonist and antagonist, respectively, are the best known agents of this type, (ii) small peptides identified by screening of synthetic peptide combinatorial libraries such as peptide III-BTD[10] and the hexapeptides (e.g., Ac-RYYRWK-NH_2) identified by Dooley et al.,[11] and (iii) N/OFQ related peptides identified by classical structure−activity relationship approaches.

We and other groups have performed a detailed series of SAR studies that allowed identification of (i) N/OFQ-(1-13)-NH_2 as the shortest N/OFQ sequence maintaining full biological activity,[12,13] (ii) [Phe$^1\Psi$(CH_2NH)Gly^2]N/OFQ(1-13)-NH_2, which is a selective partial agonist at NOP,[14−16] (iii) [Nphe^1]N/OFQ(1-13)-NH_2, a low-potency pure antagonist,[17,18] and more recently (iv) [(pF)Phe^4]N/OFQ(1-13)-NH_2, a highly potent full agonist for NOP.[19−21] Moreover, similar studies performed by the group of Shimohigashi led to the identification of [Arg^{14},Lys^{15}]N/OFQ as another highly potent NOP agonist.[22]

In the present study, we combined in the N/OFQ-NH_2 structure the chemical modifications that reduce ([Phe$^1\Psi$-(CH_2NH)Gly^2]) or eliminate ([Nphe^1]) agonist efficacy with those that increase agonist potency, i.e., [(pF)Phe^4] and [Arg^{14},Lys^{15}]. Thus, three series of four peptides each were synthesized and assessed for their in vitro pharmacological features at recombinant and native NOP receptors.

Results and Discussion

As shown in Figure 1, the compounds of the first series (**a** series) have a natural Phe^1-Gly^2 bond, those of the second series (**b** series) have a Phe$^1\Psi$(CH_2NH)-Gly^2 pseudopeptide bond, and those of the third (**c**

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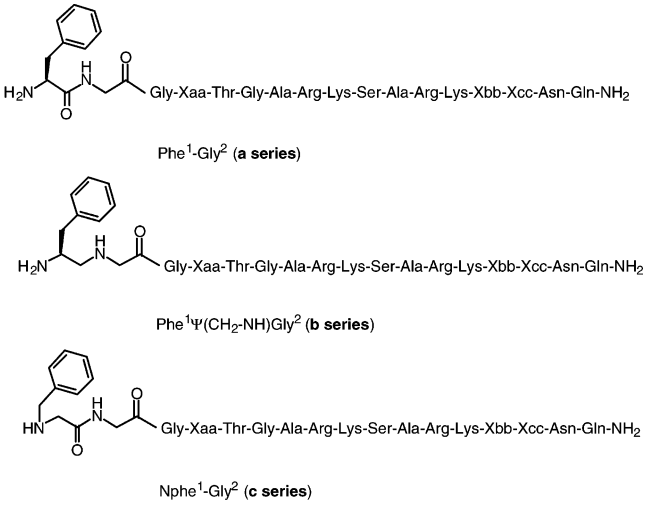

Xaa = Phe; Xbb = Leu; Xcc = Ala; Compounds **1a-c**

Xaa = (pF)Phe; Xbb = Leu; Xcc = Ala; Compounds **2a-c**

Xaa = Phe; Xbb = Arg; Xcc = Lys; Compounds **3a-c**

Xaa = (pF)Phe; Xbb = Arg; Xcc = Lys; Compounds **4a-c**

Figure 1. Chemical structures of compounds **1a**−**4a**, **1b**−**4b**, and **1c**−**4c**.

Table 1. Abbreviated Name of the N/OFQ Analogues Employed in This Study

compd	abbreviated name
1a	N/OFQ-NH_2
2a	[(pF)Phe^4]N/OFQ-NH_2
3a	[Arg^{14},Lys^{15}]N/OFQ-NH_2
4a	[(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2
1b	[Phe$^1\Psi$(CH$_2$NH)Gly^2]N/OFQ-NH_2
2b	[Phe$^1\Psi$(CH$_2$NH)Gly^2,(pF)Phe^4]N/OFQ-NH_2
3b	[Phe$^1\Psi$(CH$_2$NH)Gly^2,Arg^{14},Lys^{15}]N/OFQ-NH_2
4b	[Phe$^1\Psi$(CH$_2$NH)Gly^2,(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2
1c	[Nphe^1]N/OFQ-NH_2
2c	[Nphe^1,(pF)Phe^4]N/OFQ-NH_2
3c	[Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2
4c	[Nphe^1,(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2

series) have a peptoid (Nphe^1-Gly^2) structure. For each series we considered the natural sequence (**1**), substitution of Phe^4 with (pF)Phe^4 (**2**), substitution of Leu^{14}-Ala^{15} with Arg^{14}-Lys^{15} (**3**), and the combination of these last two modifications (**4**).

The 12 peptides were tested for their ability to bind to the receptors (Table 2) and stimulate GTPγS binding (Table 3) in Chinese hamster ovary (CHO) cells expressing the human NOP receptor (CHO_{hNOP}) membranes and to inhibit forskolin stimulated cAMP levels in CHO_{hNOP} whole cells (Table 4). Moreover, they were also tested for their ability to affect electrically evoked contraction (twitch response) of the mouse vas deferens (Table 5) and the guinea pig ileum (Table 6), two pharmacological preparations sensitive to N/OFQ.[23] In addition, Table 7 summarizes the value of affinity/potency of the compounds of the three series relative to peptides **1** in the various assays.

Receptor binding data, summarized in Table 2, demonstrated that both (pF)Phe^4 and Arg^{14}-Lys^{15} substitution increase receptor binding affinity of the N/OFQ sequence (**a** series) by 2- and 7-fold, respectively. These results confirm previous published data.[19,22] Very similar results were also obtained by applying the same modifications in the **b** and **c** series. The combination of the two chemical modifications in the same molecule produced a further increase in NOP affinity. This increase in potency compared to the reference compound **1** was additive for the **a** and **b** series ($2 + 7 \approx 10$ and $2 + 4 = 6$, respectively) but synergistic for the **c** series ($2 + 3 \ll 16$). The additive/synergistic effects on ligand affinity produced by the two chemical modifications suggest that they modulate the ligand/receptor interaction by distinct mechanisms. Some evidence supporting this view is present in the literature. In fact, QSAR studies[19] demonstrated that the increase in affinity produced by (pF)Phe^4 depends on its electron-withdrawing properties that probably facilitate, according to the model proposed by Topham et al.,[24] the interaction with the two aromatic residues Phe^{220} (TM V) and Tyr^{131} (TM III) of the NOP receptor. In contrast, the basic Arg-Lys residues of N/OFQ may promote NOP binding via electrostatic interactions with the acidic residues Asp^{195}, Glu^{194,196,197} located in the second extracellular loop of the receptor.[24] Thus, different regions of the receptor and molecular mechanisms seem to be involved in the gain of affinity produced by the (pF)Phe^4 and Arg^{14}-Lys^{15} chemical modifications of the N/OFQ sequence. As far as the functional assays are concerned, we will present and discuss separately the effects of the chemical modifications on ligand potency and on ligand efficacy.

The effects of single (**2** and **3**) and combined (**4**) chemical modifications upon binding affinity parallel those upon ligand potency obtained in the various functional assays (Tables 3−6). In fact, the (pF)Phe^4 and Arg^{14}-Lys^{15} substitutions always produce NOP ligands more potent than the reference peptides (**1**). These increases in potency were in the 2- to 50-fold range. Similar to the binding data, the combination of the two chemical modifications in the same molecule produced a further increase in ligand potency. However, these

Table 2. Receptor Binding Affinities of Compounds **1a**−**4a**, **1b**−**4b**, and **1c**−**4c** at Recombinant Human NOP Expressed on CHO Cells[a]

	pK_i ($CL_{95\%}$)		
compd [Xaa^4,Xbb^{14},Xcc^{15}]	Phe^1-Gly^2 (**a** series)	Phe$^1\Psi$(CH$_2$NH)Gly^2 (**b** series)	Nphe^1-Gly^2 (**c** series)
1 [Phe^4,Leu^{14},Ala^{15}]	10.31 (10.20−10.42)	9.89 (9.54−10.24)	9.38 (9.36−9.41)
2 [(pF)Phe^4,Leu^{14},Ala^{15}]	10.66 (10.35−10.96)	10.32 (10.17−10.47)	9.62 (9.56−9.69)
3 [Phe^4,Arg^{14},Lys^{15}]	11.16 (10.83−11.49)	10.50 (10.39−10.62)	9.89 (9.84−9.94)
4 [(pF)Phe^4,Arg^{14},Lys^{15}]	11.32 (11.06−11.59)	10.70 (10.55−10.85)	10.60 (10.16−11.04)

[a] Data are mean of at least three separate experiments. 95% confidence limits are shown in parentheses.

Table 3. Potencies of Compounds **1a**−**4a**, **1b**−**4b**, and **1c**−**4c** at Recombinant Human NOP Expressed in CHO Cells: GTPγS Binding Assay[a]

	Phe^1-Gly^2 (**a** series)			Phe$^1\Psi$(CH$_2$NH)Gly^2 (**b** series)			Nphe^1-Gly^2 (**c** series)		
compd [Xaa^4,Xbb^{14},Xcc^{15}]	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)
1 [Phe^4,Leu^{14},Ala^{15}]	8.98 (8.79−9.16)	10.98 ± 1.30	ND	8.28 (8.10−8.46)	4.85 ± 0.52	ND	inactive		7.54 (6.86−8.22)
2 [(pF)Phe^4,Leu^{14},Ala^{15}]	9.51 (9.12−9.90)	11.42 ± 0.72	ND	9.09 (9.00−9.19)	4.30 ± 0.28	ND	8.23 (7.66−8.81)	1.36 ± 0.11	8.32 (8.24−8.41)
3 [Phe^4,Arg^{14},Lys^{15}]	9.85 (9.57−10.13)	9.70 ± 0.69	ND	9.03 (8.76−9.30)	5.29 ± 0.27	ND	inactive		9.13 (8.14−10.13)
4 [(pF)Phe^4,Arg^{14},Lys^{15}]	10.12 (10.00−10.24)	12.26 ± 0.39	ND	9.68 (9.37−10.00)	4.19 ± 0.07	ND	9.39 (8.52−10.25)	2.03 ± 0.01	9.71 (9.54−9.87)

[a] For pEC$_{50}$ and pK_B values the 95% confidence limits are given in parentheses. E_{max} ± SEM values are expressed as stimulation factor (ligand specific DPM/basal specific DPM). ND: not determined because these compounds behave as full agonists. Inactive: inactive up to 10 μM. The antagonistic properties of these compounds were tested using N/OFQ as the agonist. Their potencies were assessed by testing the peptides at 1 μM (**1c** and **2c**) or 0.1 μM (**3c** and **4c**). These data are the mean of three to four separate experiments.

Table 4. Potencies of Compounds **1a**−**4a**, **1b**−**4b**, and **1c**−**4c** at Recombinant Human NOP Expressed in CHO Cells: cAMP Assay[a]

	Phe^1-Gly^2 (**a** series)			Phe$^1\Psi$(CH$_2$NH)Gly^2 (**b** series)			Nphe^1-Gly^2 (**c** series)		
compd [Xaa^4,Xbb^{14},Xcc^{15}]	agonist pEC$_{50}$	E_{max}	antagonist pK_b	agonist pEC$_{50}$	E_{max}	antagonist pK_b	agonist pEC$_{50}$	E_{max}	antagonist pK_b
1 [Phe^4,Leu^{14},Ala^{15}]	9.94 (9.82−10.07)	103 ± 1.4	ND	9.12 (8.73−9.51)	97 ± 8.6	ND	inactive		6.81 (6.39−7.23)
2 [(pF)Phe^4,Leu^{14},Ala^{15}]	10.18 (9.88−10.48)	101 ± 1.3	ND	9.32 (8.79−9.85)	104 ± 4.1	ND	7.26 (4.65−9.87)	28 ± 9.9	ND
3 [Phe^4,Arg^{14},Lys^{15}]	10.00 (9.70−10.30)	102 ± 1.0	ND	9.62 (9.26−9.98)	89 ± 9.4	ND	inactive		7.11 (6.95 −7.27)
4 [(pF)Phe^4,Arg^{14},Lys^{15}]	10.17 (9.93−10.41)	104 ± 1.1	ND	9.52 (9.00−10.04)	92 ± 2.3	ND	8.31 (7.84−8.77)	91 ± 9.5	ND

[a] For pEC$_{50}$ and pK_B values the 95% confidence limits are given in perentheses. E_{max} ± SEM values are expressed as percent inhibition of forskolin stimulated cAMP formation. ND: not determined because these compounds behave as full agonists. Inactive: inactive up to 10 μM. The antagonistic properties of these compounds were tested using N/OFQ as the agonist. Their potencies were assessed by testing the peptides at 1 μM. These data are the mean of three to five separate experiments.

Table 5. Potencies of Compounds **1a**−**4a**, **1b**−**4b**, and **1c**−**4c** at NOP Receptors Expressed in the Electrically Stimulated Mouse Vas Deferens[a]

	Phe^1-Gly^2 (**a** series)			Phe$^1\Psi$(CH$_2$NH)Gly^2 (**b** series)			Nphe^1-Gly^2 (**c** series)		
compd [Xaa^4,Xbb^{14},Xcc^{15}]	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)
1 [Phe^4,Leu^{14},Ala^{15}]	8.27 (8.11−8.43)	95 ± 2%	ND	variable agonist effects		7.17 (6.87−7.47)	inactive		6.07 (5.92−6.22)
2 [(pF)Phe^4,Leu^{14},Ala^{15}]	8.59 (8.30 −8.88)	92 ± 1%	ND	variable agonist effects		7.90 (7.65−8.15)	variable agonist effects		6.61 (6.37−6.86)
3 [Phe^4,Arg^{14},Lys^{15}]	9.12 (8.93 −9.31)	95 ± 1%	ND	variable agonist effects		8.04 (7.75−8.33)	inactive		7.24 (7.03−7.45)
4 [(pF)Phe^4,Arg^{14},Lys^{15}]	9.36 (9.14−9.58)	94 ± 1%	ND	8.99 (8.73−9.25)	53 ± 3%	9.20 (9.00−9.40)	variable agonist effects		7.97 (7.79−8.15)

[a] For pEC$_{50}$ and pK_B values the 95% confidence limits are given in parentheses. E_{max} ± SEM values are expressed as percent inhibition of electrically induced twitches. ND: not determined because these compounds behave as full agonists. Inactive: inactive up to 10 μM. Variable agonist effects indicate that a clear reduction of electrically induced contraction (never exceeding 50% of control twitches) was evident only in some (approximately 50%) of the tissues tested. The antagonistic properties of these compounds were tested using N/OFQ as the agonist. Their potencies were assessed by testing the peptides at 10 μM (**1c**), 1 μM (**1b**−**3b**, **2c**, and **3c**), or 0.1 μM (**4b** and **4c**). None of the effects of these compounds were affected by 1 μM naloxone. These data are the mean of five to eight experiments.

increases were higher for the **c** series peptides (34- to 148-fold) compared to the **b** (3- to 107-fold) and especially the **a** series (2- to 13-fold) (Table 7). These findings suggest that the combination of (pF)Phe^4 and Arg^{14}-Lys^{15} substitutions is more effective in increasing NOP ligand potency when applied to the Nphe^1-Gly^2 template than to the Phe$^1\Psi$(CH$_2$NH)Gly^2 and natural templates. However, it is worth mentioning that the potency of compound **1a** in the different assays (8.3−9.9) is higher than that of compound **1b** (6.6−9.1) and especially that of compound **1c** (6.1−7.5). Therefore, these differences in potency of the reference compounds may bias the estimation of the increase in potency caused by (pF)-Phe^4 and Arg^{14}-Lys^{15} substitutions applied in combination. Thus, we cannot firmly attribute the different abilities of the combined (pF)Phe^4 and Arg^{14}-Lys^{15} substitutions in increasing ligand potency to either the chemical structure of the template (**1a**, **1b**, **1c**) or its different potency (**1a** > **1b** > **1c**). Nevertheless, this complete series of data clearly demonstrated that the (pF)Phe^4 and Arg^{14}-Lys^{15} chemical modifications can be combined into the same molecule for generating high-potency NOP ligands.

As far as ligand efficacy is concerned, compounds of the **a** series always behave as full agonists in the various assays (Tables 3−6), indicating that the (pF)Phe^4 and Arg^{14}-Lys^{15} modifications do not change the pharmacological activity of the ligand when applied to the parent

Table 6. Potencies of Compounds **1a**−**4a**, **1b**−**4b**, and **1c**−**4c** at NOP Receptors Expressed in the Electrically Stimulated Guinea Pig Ileum[a]

	Phe[1]-Gly[2] (**a** series)			Phe[1]Ψ(CH$_2$NH)Gly[2] (**b** series)			Nphe[1]-Gly[2] (**c** series)		
compd [Xaa[4],Xbb[14],Xcc[15]]	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}, %	antagonist pK_b (CL$_{95\%}$)	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)	agonist pEC$_{50}$ (CL$_{95\%}$)	E_{max}	antagonist pK_b (CL$_{95\%}$)
1 [Phe[4],Leu[14],Ala[15]]	8.28 (8.14−8.42)	58 ± 6%	ND	variable agonist effects		6.65 (6.26−7.04)	inactive		6.21 (5.95−6.47)
2 [(pF)Phe[4],Leu[14],Ala[15]]	8.73 (8.48−8.98)	56 ± 4%	ND	variable agonist effects		8.32 (7.76−8.88)	variable agonist effects		7.16 (6.88−7.44)
3 [Phe[4],Arg[14],Lys[15]]	8.95 (8.83−9.07)	52 ± 4%	ND	variable agonist effects		7.47 (6.79−8.15)	inactive		6.79 (6.50−7.08)
4 [(pF)Phe[4],Arg[14],Lys[15]]	8.98 (8.65−9.31)	55 ± 4%	ND	variable agonist effects		8.48 (7.89−9.07)	variable agonist effects		7.82 (7.51−8.13)

[a] For pEC$_{50}$ and pK_B values the 95% confidence limits are given in parentheses. E_{max} ± SEM values are expressed as percent inhibition of electrically induced twitches. ND: not determined because these compounds behave as full agonists. Inactive: inactive up to 10 μM. Variable agonist effects indicate that a clear reduction of electrically induced contraction (never exceeding 50% of control twitches) was evident only in some (approximately 50%) of the tissues tested. The antagonistic properties of these compounds were tested using N/OFQ as the agonist. Their potencies were assessed by testing the peptides at 10 μM (**1c**), 1 μM (**1b**−**3b**, **2c**, and **3c**), or 0.1 μM (**4b** and **4c**). None of the effects of these compounds were affected by 1 μM naloxone. These data are the mean of five to eight experiments.

Table 7. Summary of the Values of Affinity/Potency of the Compounds of the **a**−**c** Series Relative to Peptides **1** in the Various Assays

	CHO$_{hNOP}$ membranes receptor binding	CHO$_{hNOP}$ membranes GTPγS binding	CHO$_{hNOP}$ cells cAMP levels	mouse vas deferens bioassay	guinea pig ileum bioassay
1a	1	1	1	1	1
2a	2	3	2	2	3
3a	7	7	1	7	5
4a	10	13	2	12	5
1b	1	1	1	1	1
2b	2	6	2	5	50
3b	4	6	3	7	7
4b	6	25	3	107	68
1c	1	1	1	1	1
2c	2	6	3	3	9
3c	3	39	2	15	4
4c	16	148	34	79	41

sequence. The full agonist activity and high potency of compound **4a** was recently confirmed in in vivo studies where after intracerebroventricular administration it mimicked the pronociceptive effects of N/OFQ in the mouse tail withdrawal assay.[25] In this assay compound **4a** not only displayed high potency compared to N/OFQ but also showed rather long lasting effects.[25]

Similar results were obtained with the compounds of the **b** series that behaved as partial agonists, eliciting only a fraction of the N/OFQ maximal effect in the GTPγS binding (Table 3) and in the isolated tissues bioassays (Tables 5 and 6). In contrast, in the cAMP assay performed in CHO$_{hNOP}$ cells, peptides of the **b** series behaved as full agonists (Table 4), confirming previous findings obtained with [Phe[1]Ψ(CH$_2$NH)Gly[2]]N/OFQ(1-13)-NH$_2$ by us[26] and others.[27] This apparent discrepancy can probably be explained by considering the very high efficiency of the stimulus/response coupling that characterizes this particular assay. The recent demonstration that ligand efficacy strongly depends on the level of NOP receptor expression[28] corroborates this hypothesis. We have previously commented on the need to validate potency and efficacy in a range of tissues/assays,[6] and the present results further reemphasize the need for caution when using a single functional endpoint in SAR studies.

Compounds **1c** and **3c** behaved as NOP antagonists, while the presence of the (pF)Phe[4] chemical modification, as in compounds **2c** and **4c**, increased not only the ligand potency but also its efficacy, thus generating partial agonists (Tables 3−6). This increase of ligand efficacy appears to be more important when the (pF)-Phe[4] chemical modification is associated with the Arg[14]-Lys[15] substitution. Indeed, in the cAMP assay compound **2c** behaved as a partial agonist (intrinsic activity of ∼0.3) while compound **4c** behaved almost as a full agonist (intrinsic activity of ∼0.9). Therefore, although in terms of potency the (pF)Phe[4] and Arg[14]-Lys[15] modifications applied to the Nphe[1]-Gly[2] template (**c** series) produced the same effects (**4c** > **3c** = **2c** > **1c**) observed with the compounds of the **a** and **b** series, in terms of ligand efficacy the (pF)Phe[4] and Arg[14]-Lys[15] produced different changes, the former being able to increase this parameter. Thus, only the Arg[14]-Lys[15] modification can be used for increasing the antagonist potency of [Nphe[1]]N/OFQ without modifying its pharmacological behavior. The antagonist properties of compound **3c** were confirmed in a variety of in vitro assays including neurochemical (5-HT release from rat[29] and mouse[30] synaptosomes) and electrophysiological (N/OFQ induced currents in PAG slices[31]) studies, as well as in human tissues such as the bronchus[32] and monocytes.[33] Moreover, the NOP antagonist profile of compound **3c** was confirmed in vivo in mice evaluated for the supraspinal pronociceptive effects of N/OFQ,[29] the spinal antinociceptive effects of N/OFQ,[34] and the antidepressant-like effects of NOP antagonists,[35,36] which were recently confirmed in rats,[37] and in guinea pigs evaluated for the cardiovascular effects of N/OFQ.[38]

In conclusion, the present study led to the identification of novel peptide NOP ligands. Among the 12 peptides synthesized and assayed, the most interesting compounds were the full agonist compound **4a** ([(pF)-Phe[4],Arg[14],Lys[15]]N/OFQ-NH$_2$), the partial agonist compound **4b** ([Phe[1]Ψ(CH$_2$NH)Gly[2],(pF)Phe[4],Arg[14],Lys[15]]N/OFQ-NH$_2$), and the pure antagonist compound **3c** ([Nphe[1],Arg[14],Lys[15]]N/OFQ-NH$_2$). To date, they represent the most potent peptide ligands for the NOP receptor.

Experimental Section

1. Materials. Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, or Fluka (Switzerland). *N*-Benzylglycine was from Aldrich (Mil-

waukee WI). The resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid]poly(ethylene glycol)/polystyrene suport (Fmoc-PAL-PEG-PS) was from Millipore (Waltham, MA). Naloxone was from Tocris Cookson. Stock solutions (1 mM) of peptides were made in distilled water and kept at −20 °C until ready for use. Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) had the following composition (in mM): NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10. All other reagents were from Sigma Chemical Co. or E. Merck and were of the highest purity available.

2. Peptide Synthesis and Purification. Peptides of the **a** and **c** series were obtained as previously described[18] by stepwise solid-phase peptide synthesis and purified by using HPLC chromatography. Peptides of the **b** series, containing the reduced Phe[1]-Gly[2] peptide bond, were obtained by condensing Boc-Phe-CHO with the N/OFQ(2-17)-PAL-PEG-PS resin and reducing the intermediate imine derivative in situ with $NaBH_3CN$ as previously described.[16] Peptide synthesis and purification details are available in Supporting Information.

3. CHO_{hNOP} Cell Studies All tissue culture media and supplements were from Invitrogen (Paisley, U.K.). [Leucyl-^{3}H]N/OFQ (150−152 Ci/mmol) was from Amersham Biosciences (Buckinghamshire, U.K.), and GTPγ[^{35}S] (1250 Ci/mmol) and [2,8-^{3}H]-cAMP (28 Ci/mmol) were from Perkin-Elmer Life Sciences (MA). All other reagents were of the highest purity available.

3.1. Tissue Culture and Cell/Membrane Preparation. CHO cells stably expressing the human NOP receptor (CHO_{hNOP}) were cultured in media consisting of Dulbecco's MEM:HAMS F12 (1:1, v/v) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, 5% (v/v) fetal calf serum, 200 μg/mL geneticin (G418), and 200 μg/mL hygromycin B at 37 °C and 5% CO_2 humidified air. The antibiotics G418 and hygromycin B, which select for expression of recombinant receptor and a reporter gene, respectively, were omitted from experimental cultures. Cells were subcultured as required using trypsin/EDTA and used for experimentation once they were confluent. Membranes were prepared from freshly harvested (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1.7 mM EDTA, pH 7.4) cells in homogenizing buffer of either 50 mM Tris-HCl, 5 mM $MgSO_4$, pH 7.4 (competition binding), or 50 mM Tris-HCl, 0.2 mM EGTA (GTPγ[^{35}S] binding). Membrane fragments were prepared by homogenization (Ultra-turrax) and centrifugation (20000*g* at 4 °C for 10 min) for a total of three times. Total protein content was then determined using the method Lowry et al.[39] For cAMP assays whole cell pellets from freshly harvested cells were washed twice with and finally resuspended in Krebs/HEPES buffer of the following composition (mM): Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), $H_2PO_4^{2-}$ (1.2), SO_4^{2-} (1.2), glucose (11.7), HEPES (10) containing 0.5% bovine serum albumin.

3.2. [Leucyl-^{3}H]N/OFQ Competition Binding. An amount of 10 μg of protein of CHO_{hNOP} homogenate was assayed in a total volume of 0.5 mL consisting of competition homogenizing buffer supplemented with 0.5% (w/v) BSA, 10 μM peptidase inhibitors (amastatin, bestatin, captopril, and phosphoramidon), 0.2 nM [leucyl-^{3}H]N/OFQ, and 100 nM to 0.1 pM of competing ligands. Nonspecific binding was determined in the presence of 1 μM N/OFQ. Reaction mixtures were incubated for 1 h at room temperature and harvested under vacuum filtration through Whatman GF/B filters soaked in 0.5% polyethylenimine. Radioactivity was determined after 8 h of extraction in scintillation cocktail ("Optiphase Safe").

3.3. GTPγ[^{35}S] Binding. An amount of 20 μg of CHO_{hNOP} homogenate was incubated in a 0.5 mL assay buffer consisting of 50 mM Tris-HCl, 1 mM $MgCl_2$, 100 mM NaCl, and 0.2 mM EGTA supplemented with 1 mg/mL BSA, 150 μM bacitracin, 10 μM peptidase inhibitors (as above), 100 μM GDP, 150 pM GTPγ[^{35}S], and ligands at varying concentrations and in varying combinations. Nonspecific binding was defined in the presence of 10 μM GTPγS. Reaction mixtures were incubated for 1 h at 30 °C with gentle shaking, and the reaction was terminated under vacuum filtration through Whatman GF/B filters. Polyethylenimine was not used. Radioactivity was determined as for competition binding.

3.4. cAMP Cell Suspension Assay. Confluent cell cultures were harvested, and the pellet was resuspended in assay buffer (0.5% BSA Krebs/HEPES). The cell suspension was incubated at 37 °C for 15 min in the presence of 1 mM isobutylmethylxanthine, 1 μM forskolin, and ligand at varying concentrations in a total volume of 300 μL. Basal level was determined in the absence of forskolin and ligand. Protease and peptidase inhibitors were not necessary for this assay. The assay was stopped by HCl (10 M), neutralized with NaOH (10 M), and buffered to pH 7.4 by Tris-HCl (1 M). Cellular debris was cleared by centrifugation. The supernatant was incubated at 4 °C for at least 2.5 h in a competitive binding assay with tritiated cAMP and cAMP binding protein (see ref 28 for details).

4. Bioassay Studies. Male Swiss mice weighing 25−30 g and albino guinea pigs weighing 250−300 g were used. The bioassay experiments were performed as previously described.[23] The mouse vas deferens and guinea pig ileum tissues were suspended in 5 mL organ baths containing Krebs solution. For mouse vas deferens experiments the bath temperature was set at 33 °C and the Krebs solution was Mg^{2+}-free, while for guinea pig ileum experiments the bath temperature was set at 37 °C. The tissues were stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. The resting tension was maintained at 0.3 g. The electrically evoked contractions were measured isotonically by means of a Basile strain gauge transducer and recorded with the a PC-based acquisition system (Autotrace, RCS, Florence, Italy). After an equilibration period of about 1 h the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration−response curves to N/OFQ and related peptides were performed (0.5 log unit steps). When required, antagonists were added to the Krebs solution 15 min before performing the concentration−response curve to N/OFQ.

5. Data Analysis. The pharmacological terminology adopted in this study is in line with IUPHAR recommendations:[40] the agonist potencies are given as pEC_{50}, which is the negative logarithm to the base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. Antagonist potencies have been evaluated using the Gaddum−Schild equation: $pK_b = \log((CR - 1)/[\text{antagonist}])$, assuming a slope value equal to unity and where CR indicates the ratio of agonist potency in the presence of antagonist to that in the absence of antagonist. The ligand affinities obtained in binding competition experiments are given as pK_i, which is the negative logarithm to the base 10 of the inhibition equilibrium constant. Data were analyzed using nonlinear curve fitting with variable slopes for derivation of pEC_{50} and IC_{50} values (GraphPad Prism V3, San Diego, CA). The pK_i values were calculated using the Cheng and Prusoff equation, $\log[IC_{50}/([L]/K_D + 1)]$, where IC_{50} is the concentration of competitor producing 50% displacement and where K_D is assumed to be 70.8 pM for [^{3}H]N/OFQ measured previously in CHO_{hNOP} cell membranes.[41] GTPγ[^{35}S] binding data are presented as stimulation factors (ligand specific DPM/basal specific DPM), and cAMP data are presented as the percentage inhibition of forskolin stimulated cAMP formation.

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Supporting Information Available: General procedures for the solid-phase peptide synthesis and for on-resin synthesis

of the reduced Phe[1]-Gly[2] peptide bond, peptide purification procedures, and a table reporting the analytical properties of the peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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