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Identification of an achiral analogue of J-113397 as potent nociceptin/orphanin FQ receptor antagonist

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Abstract—To date, J-113397 represents the most potent and selective non peptide NOP receptor antagonist widely used in pharmacological studies. However, the synthesis, purification, and enantiomer separation of this molecule, which contains two chiral centers, is rather difficult and low-yielding. Here, we synthesized and tested a series of simplified J-113397 analogues to investigate the importance of the stereochemistry and the influence of the substituents at position 3 of the piperidine nucleus and on the nitrogen atom of the benzimidazolidinone nucleus. The compound coded as Trap-101, an achiral analogue of J-113397, combines a pharmacological profile similar to that of the parent compound with a practical, high-yielding preparation.

1. Introduction

The nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP, [1]) was cloned 10 years ago as a novel member of the opioid family of receptors. However, it was soon evident that despite the close structural and transductional similarities the NOP receptor does not bind opioid ligands and displays a unique pharmacology.[2,3] The NOP receptor was then used for identifying its endogenous ligand, the neuropeptide N/OFQ,[4,5] the first successful example of reverse pharmacology.[6] Thereafter, the N/OFQ-NOP receptor system has been shown to be involved in the regulation of a variety of central and peripheral functions, however, most of the early studies were performed simply administering N/OFQ.[2,3] For deeply understanding the biological roles of the N/OFQ-NOP receptor system potent and selective antagonists, possibly of non-peptide nature, are required. The first reported molecule displaying such features was J-113397[7] (Chart 1), which was shown to bind with nanomolar affinity to NOP receptors and to display 100- to 300-fold selectivity over classical opioid receptors.[8,9] J-113397 antagonized N/OFQ effects at human NOP in a competitive manner with pA_2 values in the range of 7.5–8.9 in cAMP and GTPγS assays.[9,10] The selective antagonist properties of J-113397 were confirmed at native NOP receptors expressed in isolated tissues[11–14] and in brain preparations evaluated with biochemical,[15–17] neurochemical,[18–20] and electrophysiological[21–24] techniques. J-113397 was also investigated in vivo where, in the range of 1–30 mg/kg, it prevented the actions of N/OFQ on pain transmission,[9,25,26] on airways[27] and cough reflex,[28,29] and on gastrointestinal functions.[14,30] Moreover, J-113397 produced per se pronociceptive effects in the rat[31] and mouse[32] formalin test, antidepressant-like effects (similar to NOP receptor peptide antagonists[33,34]) in the forced swimming test,[33] reduction of kainate-induced seizures,[35] and potentiation of buprenorphine analgesia in wild type but not in NOP knockout mice,[36] and facilitation of striatal dopamine release and locomotor performance on the rotarod in rats.[37] This latter effect was recently confirmed in 6-hydroxydopamine lesioned animals.[38]

Keywords: Nociceptin/orphanin FQ; NOP receptor; Non peptide antagonists; J-113397; Mouse vas deferens assay; Structure–activity study.
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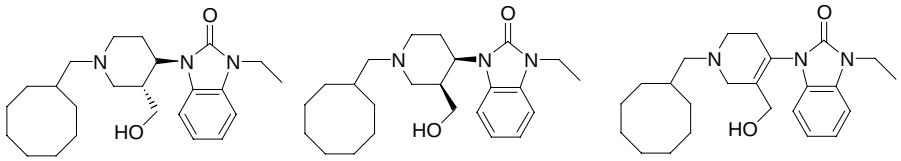


J-113397 compound **1** Trap- 101

Chart 1. Structure of the compounds selected for molecular modeling studies.

The use of J-113397 has been limited by the lack of commercial availability of the compound and the difficulties related to the synthesis and purification of a compound that contains 2 chiral centers. In the recent past, we were not able to replicate the synthetic approach described by Kawamoto[7,39] and we developed an original alternative synthesis for J-113397[40] which, however, revealed operational issues associated with low yields in the last steps and purification.

The present study was performed in an attempt to identify J-113397 derivatives with a similar pharmacological profile but obtainable with shorter/high-yielding synthetic procedures. We first considered *cis*- and *trans*-J-113397 as racemic compounds to evaluate the influence of the chirality at C3–C4 carbon atoms of the piperidine ring system. Then we decided to simplify the molecule removing the two chiral centers through the introduction of a C3–C4 double bond. Interestingly, the derived compound, coded as Trap101 (Chart 1), displayed a pharmacological activity similar to that of (±)J-113397 and could be used as a novel template for a structure–activity study aimed at establishing the importance of both the C3 hydroxymethyl function and of the benzimidazolidinone nitrogen substituent. All new compounds were pharmacologically evaluated in the electrically stimulated mouse vas deferens[41] using N/OFQ as NOP receptor agonist.

2. Chemistry

Scheme 1 reports the synthesis of (±)J-113397 and Trap-101 analogues starting from compound **I**, as described by De Risi et al.[40] $LiAlH_4$ reduction of compound **I** under mild conditions (15 min at 0 °C) allowed us to obtain compound **7** preserving the carbamate moiety at position N3 of benzimidazolidinone. Removal of the nitrogen protective group of **I** by treatment with TFA in DCM at rt gave the free amide **II**, which was transformed to **6** by $LiAlH_4$ reduction of the ester group at position C3. Alkylation of **II** with ethylbromide or ethylbromoacetate in DMF, in the presence of sodium hydride, gave compounds **5** and **III**, respectively. It is noteworthy that the key intermediate **5**, bearing all the functionalities required to obtain Trap-101, its analogues as well as (±)J-113397 analogues, could be produced in a multigrams scale with a multistep high-yielding reactions. $LiAlH_4$ reduction of **5** produced Trap-101, while that of **III** proceeded with concomitant reduction of both ester groups, leading efficiently to the formation of compound **8**.

High pressure (1000 psi) catalytic hydrogenation of **5** produced the *cis* isomer **3** with increased yield compared to the published reaction conditions.[40] The *trans* isomer **2** was easily obtained by treatment of **3** with MeONa in anhydrous methanol at rt for 60 h, while $LiAlH_4$ reduction of **3** in THF at 0 °C for 15 min gave the *cis*-alcohol **1**.

The *trans*-ester **2** was used to obtain both (±)J-113397 and compound **4**, simply by increasing the reduction time from 15 to 45 min.

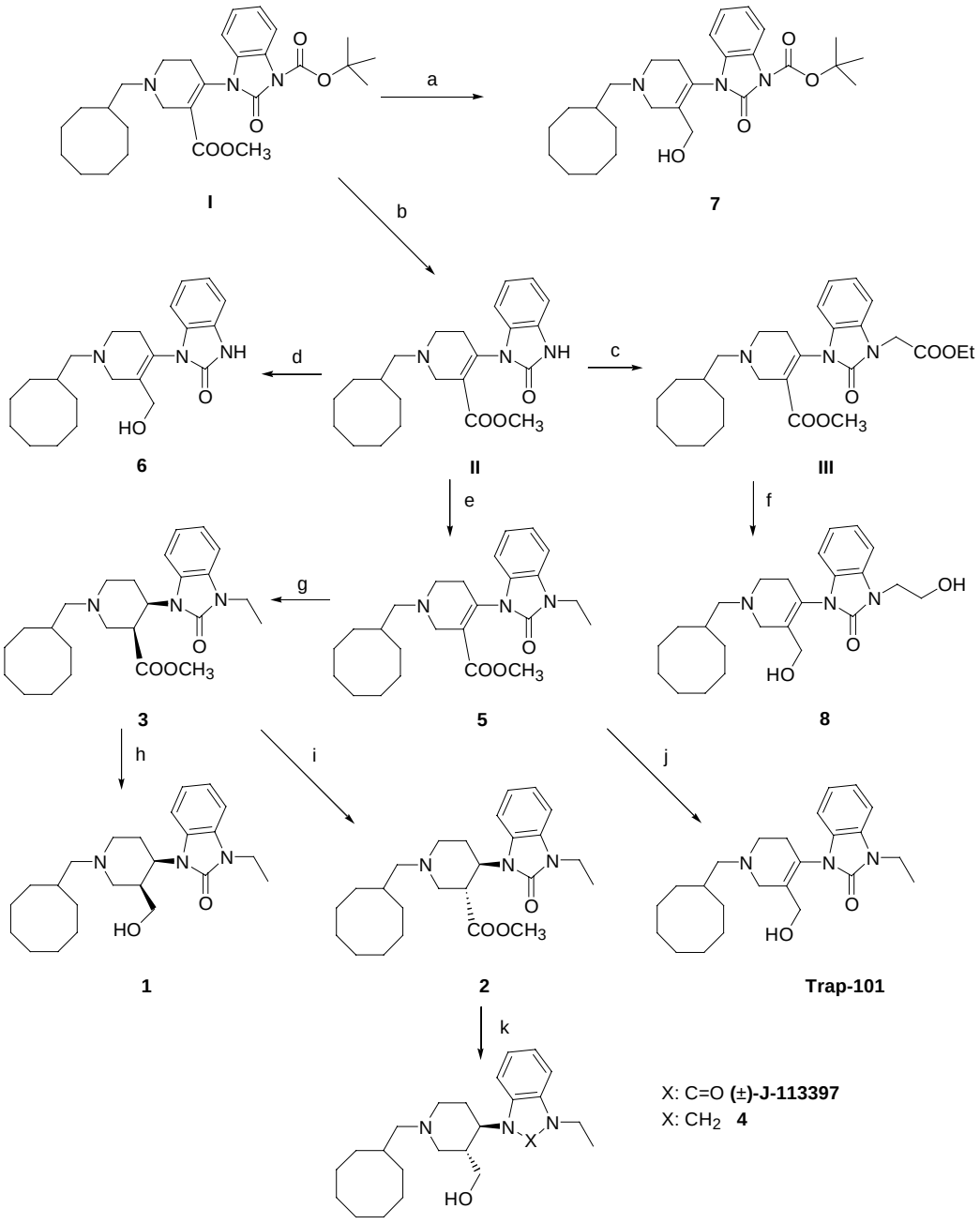
The synthesis of compound **9** is depicted in Scheme 2. Double Michael addition of the cycloctenmethylamine **IV**, easily prepared by reduction of 1-nitromethyl-cyclooctene,[42] to methyl acrylate produces the adduct **V** which underwent Dieckmann cyclization promoted by ^{t}ButOK to furnish the desired β-ketoester **VI**.

Heating of a mixture of β-ketoester **VI** and *o*-phenylenediamine in toluene at reflux in the presence of a catalytic amount of AcOH and 4 A molecular sieves yielded the stable enamine **VII**, which on treatment with an excess of di-*tert*-butyldicarbonate and DMAP led to the formation of the benzimidazolidinone nucleus protected on the nitrogen N3 as the Boc-derivative **VIII**.

The deprotection of compound **VIII** with TFA in DCM and the alkylation at the N3 nitrogen of the benzimidazolidinone with ethyl bromide in the presence of NaH furnished **IX** that was transformed to **9** by reduction of the ester moiety with $LiAlH_4$.

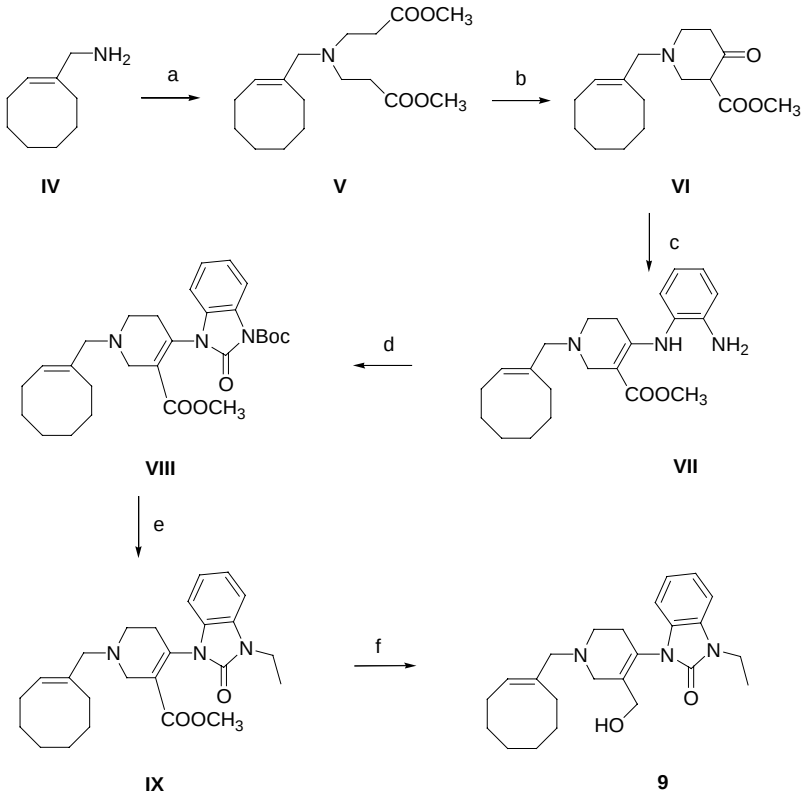
3. Molecular modeling studies

An effort was made for exploring the conformational features of the structures of compounds J-113397, compound **1**, and Trap-101 (Chart 1). Assuming that all ligands bind at the same site of the receptor, they should adopt a common three-dimensional geometry that is responsible for their biological activity. Since the absolute configuration of the active enantiomer (3R,4R)J-113397 was determined by X-ray crystallography[39] (CCDC 151492), we used this information as a starting point for further conformational analyses performed on the three compounds. In MM calculations, no solvent was taken into account and semiempirical geometry reoptimization, using the AM1 method,[43] was performed. The lowest energy structure of J-113397, generated in the conformational search, has an opposite orientation of the benzimidazolidinone group



Scheme 1. Synthesis of (±)J-113397, Trap-101, and compounds **1**–**8**. Reagents: (a) $LiAlH_4$, THF, 0 °C to rt, 15 min; (b) TFA, DCM, 0 °C to rt, 8 h; (c) $BrCH_2COOEt$, NAH, DMF, 0 °C to rt, 12 h; (d) $LiAlH_4$, THF, 0 °C to rt, 15 min; (e) EtBr, NAH, DMF, 0 °C to rt, 12 h; (f) $LiAlH_4$, THF, 0 °C to rt, 30 min; (g) H_2 60 atm, 10% C/Pd, MeOH, 24 h; (h) $LiAlH_4$, THF, 0 °C to rt, 15 min; (i) MeONa, MeOH, rt, 60 h; (j) $LiAlH_4$, THF, 0 °C to rt, 15 min; (k) $LiAlH_4$, THF, 0 °C to rt, 15 min to (±)-J113397, 45 min to 4.

compared to the crystal structure,[39] however, the energy difference of the two conformers was negligible (0.05 kcal/mol). The bioactive conformation of J-113397 was chosen according to crystal structure and the results of previous molecular modeling studies, [44] in which a 3D-QSAR analysis on a set of piperidine based NOP agonists was performed. To select the bioactive conformer, compound **1** and Trap-101 were superimposed on the reference compound J-113397 by matching the common correspondent piperidine ring and the centroids (the geometrical centers) of the cyclooctyl ring and benzimidazolidinone system (Fig. 1). The smallest RMSD of the fitting procedure and the energy values (kcal) were used as parameters. The selected conformation of Trap-101 fits with a minimum RMSD = 0.250 Å and has a energy of 1.770 kcal/mol above the lowest energy minima calculated in the conformational analysis. In the case of compound **1**, both the enantiomers (3*R*,4*S*; 3*S*,4*R*) were taken into account. The selected low energy conformations of enantiomers 3*R*,4*S* and 3*S*,4*R*, fit with a minimum RMSD of 0.951 and 0.675 Å, respectively, with the reference compound J-113397. Enantiomer 3*S*,4*R* was chosen as bioactive structure according to the results of the



Scheme 2. Synthesis of compound **9**. Reagents: (a) CH_2=CH–$COOCH_3$, MeOH, rt, 12 h; (b) ^{t}ButOK, toluene, 0°C to rt, 12 h; (c) *o*-phenylenediamine, AcOH, MS 4A, toluene at reflux, 12 h; (d) $(Boc)_2O$, DMAP, DCM, 0 °C, 3 h; (e) (1) TFA, DCM, 0 °C to rt, 8 h; (2) EtBr, NaH, DMF, 0 °C to rt, 8 h; (f) $LiAlH_4$, THF, 0 °C, 15 min.

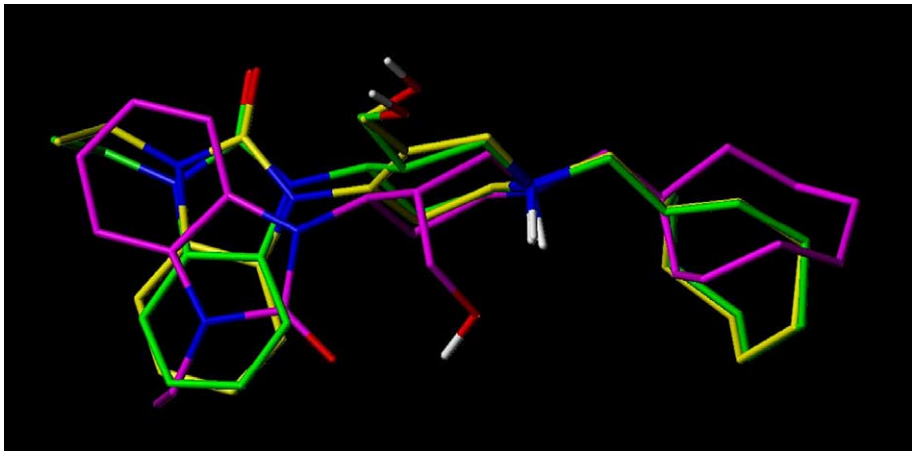


Figure 1. Superimposition of compounds **1** (purple) and Trap-101 (yellow) on the reference molecule J-113397 (green).

alignment procedure. All the calculated low energy conformations of enantiomer 3*S*,4*R* showed two intramolecular hydrogen bonds: one between NH of piperidine ring and the OH of the hydroxymethyl function, and one between the OH and the O of the benzimidazolidinone moiety. The lowest energy conformation of compound **1** (3*S*,4*R*), selected in the molecular superimposition, is characterized by an interatomic distance between the hydrogen of the piperidine nitrogen and the oxygen of the hydroxymethyl group of 2.07 Å (angle N-H...O = 128.38°), and an interatomic distance between the hydrogen of the hydromethyl group and the oxygen of the benzimidazolidinone moiety of 2.120 Å (angle O-H...O = 131.71°).

4. Results and discussion

All compounds were tested in the electrically stimulated mouse vas deferens, a pharmacological preparation sensitive to N/OFQ,[41] currently used for characterizing large series of NOP ligands,[3,45] including (±)J-113397, which behaved as a potent and competitive NOP receptor antagonist showing a pA_2 value of 7.89.[11]

Results obtained with the new compounds have been summarized in Table 1. None of the compounds showed agonist activity up to 10 μM. N/OFQ produced a concentration dependent inhibition of the electrically induced twitch response with a pEC_{50} of 7.72 and an E_{max} of −89%.

In line with previous findings,[11] the reference compound (±)J-113397 produced a rightward parallel shift of the concentration–response curve to N/OFQ without modifying the maximal effect of the peptide; a pA_2 value of 7.79 was calculated from these experiments (Table 1). The cis isomer compound **1** also behaved as an antagonist showing, however, 100-fold lower potency (pA_2 5.88). This suggests that the stereochemistry of C3–C4 carbon atoms is highly important for biological activity, with the cis conformation limiting NOP receptor occupation. Other NOP receptor ligands characterized by a piperidine 1,4 disubstituted scaffold, such as the agonists Ro-65-6570[8,46] and Ro 64-6198,[47,48] and the recently discovered antagonist SB-612111[49] contain chiral centers which are important for receptor binding; however, in these molecules the chirality is on the piperidine nitrogen substituent and not on the piperidine carbon atoms.

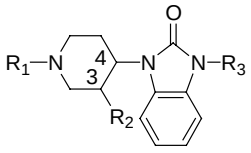
To investigate the relative importance of the hydroxymethyl function in position 3 of the piperidine nucleus for NOP receptor interaction, we evaluated compounds **2** and **3** which have a methyl ester instead of the primary alcoholic function. Compound **2** was completely inactive up to 10 μM, while compound **3** showed a pA_2 value of 6.18, similar to that of compound **1**. The very different biological effect of the methyl ester function in position 3 suggests that the spatial orientation of the primary alcoholic function of J-113397 is crucial for its biological activity.

The reduction of the carbonyl function of the benzimidazolidinone nucleus (compound **4**) generated a (±)J-113397 analogue equipotent to the parent molecule indicating that the oxygen atom is not relevant for NOP receptor occupation.

To further investigate the importance of the chirality at C3–C4 carbon atoms, we removed it introducing a double bond, thus generating Trap-101 (Chart 1). The new achiral J-113397 derivative behaved as a pure NOP receptor antagonist with a pA_2 value (7.75) superimposable to that of the reference compound. This result, together with those obtained with (±)J-113397 and compound **1**, suggests that the chirality of the C3–C4 piperidine atoms is important but not essential for NOP receptor interaction. This is corroborated by the findings by Kawamoto et al.[7] who, by eliminating the hydroxymethyl function in position 3, generated an achiral analogue of J-113397 (compound 15 in the Kawamoto paper) which maintained high binding affinity to the NOP receptor. These findings prompted us to perform a molecular modeling study on (±)J-113397, the corresponding cis isomer (compound 1) and the achiral analogue Trap-101, to get information regarding the spatial disposition of the piperidine substituents.

Figure 1 shows the superimposition of the piperidine and the centroids of the cyclooctyl and benzimidazolidinone moieties of **1** and Trap-101 on the reference J-113397. The benzimidazolidinone moiety of J-113397 and Trap-101 is better overlaid than that of compound **1**. The same can be said for the hydroxymethyl pharmacophore whose spatial orientation is almost identical in J-113397 and Trap-101 while very different in compound **1**. Thus, these molecular modeling results, together with those obtained by bioassay, point to a crucial role of the spatial orientation of both the benzimidazolidinone and

Table 1. Structure and mouse vas deferens activity of J-113397 and Trap-101 analogues



Compound	R_1	R_2	R_3	C_3–C_4	Antagonist[a] pA_2 ($CL_{95\%}$)
±J-113397	Cy	CH_2OH	Et	Trans	7.79 (7.45–8.13)
1	Cy	CH_2OH	Et	Cis	5.88 (5.55–6.21)
2	Cy	$COOCH_3$	Et	Trans	<5
3	Cy	$COOCH_3$	Et	Cis	6.18 (5.83–6.53)
4[b]	Cy	CH_2OH	Et (benzimidazole)	Trans	7.60 (7.30–7.90)
Trap-101	Cy	CH_2OH	Et	DB	7.75 (7.48–8.02)
5	Cy	$COOCH_3$	Et	DB	5.49 (5.02–5.96)
6	Cy	CH_2OH	H	DB	6.79 (6.44–7.14)
7	Cy	CH_2OH	Boc	DB	6.79 (6.62–6.92)
8	Cy	CH_2OH	CH_2CH_2OH	DB	6.86 (6.63–7.09)
9	CyDB	CH_2OH	Et	DB	7.02 (6.74–7.30)

$CL_{95\%}$, confidence limits 95%. The data are means of at least 5 separate experiments. Cy, cyclooctylmethyl; CyDB, 1-Cycloct-1enylmethyl.

[a] All the compounds were tested in the electrically stimulated mouse vas deferens as agonists and were found to be inactive up to 10 μM. The antagonistic properties of the compounds were evaluated using N/OFQ as agonist. N/OFQ produced a concentration dependent inhibition of the electrically induced twitch response with a pEC_{50} of 7.72 (7.57–7.87) and a maximal effect of 89 ± 1% of control twitch.

[b] In compound 4, the carbonyl group of the benzimidazolidinone moiety has been reduced.

hydroxymethyl pharmacophores (superimposable in (±)J-113397 and Trap-101, but not in compound **1**) for an optimal NOP receptor binding (high potency of (±)J-113397 and Trap-101, low potency of compound **1**). Moreover, it should be noted that an intermolecular hydrogen bond between the hydroxy group and the piperidine nitrogen is present in compound **1** but not in the other two molecules (Fig. 1); thus, the hydroxy group and the piperidine nitrogen of J-113397 and Trap-101 are available for possible hydrogen bonds with the NOP receptor, while this cannot happen (or at least is highly unlikely) with compound **1**. The importance of the hydroxymethyl group is also emphasized by the fact that compound 15 of the Kawamoto paper[7] (which has been synthesized and evaluated by us in the electrically stimulated mouse vas deferens) displayed very low potency (pA_2 6.04) compared to that of J-113397 or Trap-101.

Trap-101 was then used as a template for investigating the importance of the C3 piperidine (compound **5**) and benzimidazolidinone nitrogen (compounds **6**–**8**) substituents.

Similar to what happens with compound **2**, the insertion of a methyl ester function in position C3 of Trap-101 (compound **5**) produced a drastic decrease in potency (pA_2 5.49). This findings suggests a similar favorable role of the primary alcoholic function both in (±)J-113397 and Trap-101.

The low potency of compounds **2** and **5** could be related to the increase of the steric hindrance and/or hydrophobicity of the methyl ester group compared to the hydroxyl function. Alternatively, the primary alcoholic moiety should be available for interacting with an amino acid of the NOP receptor binding pocket. It is worthy of mention that a model of interaction between the NOP receptor and the nonpeptide NOP receptor agonist Ro 64-6198 has been recently proposed. [44] This model indicates as crucial for ligand–receptor interaction; (i) the salt bridge between the protonated piperidine nitrogen and the Asp^{130} residue of the receptor and (ii) the hydrogen bond that may be formed between the hydrogen of the amide nitrogen in position 3 of the 1,3,8-triaza-spiro[4.5]decan-4-one nucleus and the oxygen of the receptor Thr^{305} side chain. Interestingly enough, the Asp^{130} residue of the NOP receptor is also considered crucial for the interaction with the protonated N terminal of the natural ligand N/OFQ as described in the ligand–receptor model generated by Topham.[50] The replacement of the H atom in position 3 of the 1,3,8-triaza-spiro[4.5]decan-4-one nucleus with an acetic acid methyl ester function as in the NOP ligand NNC 63-0532[51] produces an important decrease in NOP affinity; as a matter of fact, the potency of NNC 63-0532 is 100-fold lower than that of N/OFQ in cAMP experiments performed in cells expressing the human NOP[51] while that of Ro 64-6198 is, in similar experiments, only 7-fold lower.[10] A similar trend of decreased potency was observed with (±)J-113397 and Trap-101 and their corresponding compounds **2** and **5**, suggesting that these molecules may also interact with the Thr^{305} residue of the NOP receptor by forming a hydrogen bond with their primary alcoholic function, while this is unfavorable for compound **1**, whose primary alcoholic function is involved in two intramolecular hydrogen bonds. Interestingly enough, both (±)J-113397 and Trap-101 also contain a basic nitrogen (the piperidine nitrogen and that of the 1,2,3,6-tetrahydro-pyridine nucleus, respectively) as in Ro-64-6198 (and NNC 63-0532), which may interact with the Asp^{130} residue of the NOP receptor by forming a salt bridge. However, it should be noted that (±)J-113397 and Trap-101 behave as pure receptor antagonists, while Ro-64-6198 and NNC 63-0532 as full agonists; thus, if our interpretations are true, both the salt bridge and hydrogen bond are important for receptor occupation but not activation. Receptor activation may depend on the substituents that are present in positions 1 and 4 of the piperidine scaffold. This view is corroborated by recent findings by Zaveri et al.[52] who reported a novel series of NOP ligands encompassing agonist and antagonist activities obtained by modification of the piperidine nitrogen substituent.

To investigate the role played by the substituent (R3) on the benzimidazolidinone nitrogen in Trap-101 biological activity, we eliminated (compound **6**) or replaced the ethyl group with the more hydrophobic, Boc (compound **7**), or the more hydrophilic, hydroxyethyl (compound **8**) moieties. Compounds **6**–**8** behaved as NOP receptor antagonists showing, however, 10-fold reduced potency. Similar results have been obtained on the J-113397 template,[7] suggesting that the best benzimidazolidinone nitrogen substituent is indeed the ethyl group. A further insertion of a double bond on the cyclooctylmethyl (R1) group, as in (compound **9**), produced a 5-fold reduction in potency compared to Trap-101.

The selectivity of action of Trap-101 over classical opioid receptors was investigated by testing the compound against the inhibitory effects of selective agonists in the electrically stimulated mouse vas deferens and guinea pig ileum, and by evaluating Trap-101 in receptor binding experiments performed on membranes of CHO cells expressing NOP or classical opioid receptors of the mu (MOP), delta (DOP), and kappa (KOP) types. Trap-101 up to 1 μM did not modify either the potency or the maximal effects induced by the DOP selective agonist deltorphin I in the mouse vas deferens (control: pEC_{50} 10.99, E_{max} 92 ± 1%; 1 μM Trap-101: pEC_{50} 11.08, E_{max} 94 ± 1%) or by the MOP selective agonist dermorphin in the guinea pig ileum (control: pEC_{50} 9.18, E_{max} 95 ± 2%; 1 μM Trap-101: pEC_{50} 9.18, E_{max} 91 ± 2%). Moreover, receptor binding experiments sum-

Table 2. Receptor binding affinities of N/OFQ, (±)J-113397 and Trap-101 at recombinant human NOP, and classical opioid receptors expressed in CHO cell membranes

	NOP	MOP	DOP	KOP
N/OFQ	9.89	6.49	<5	<5
(±)J-113397	9.15	6.98	<5	<6
Trap-101	8.65	6.60	<5	6.14

These data, expressed as pK_i values, are means of at least 3 separate experiments.

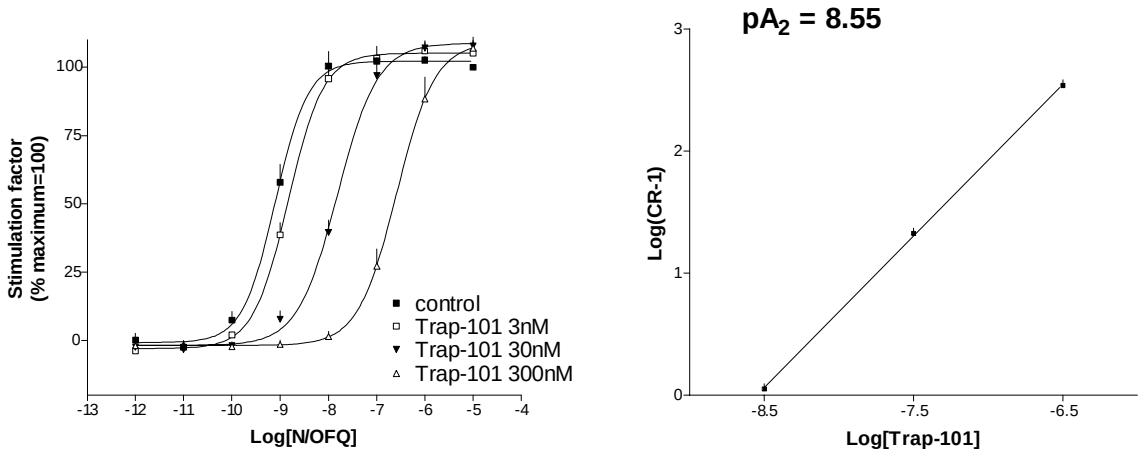


Figure 2. Left panel: effects of Trap-101 (3, 30, and 300 nM) on N/OFQ stimulated GTPγ^{35}S binding to CHO_{hNOP} membranes (stimulation factor expressed as a percentage of the maximum N/OFQ response). Trap-101 produced a concentration dependent parallel rightward shift of the concentration–response curve to N/OFQ. These data were used to generate the Schild Plot shown in the right panel. Data are means ± SEM of 4 separate experiments.

marized in Table 2 show that Trap-101 binds to the NOP receptor with high affinity only 3-fold lower than that of (±)J-113397 while maintaining a similar profile of selectivity over classical opioid receptors (approx 100-fold) as the reference molecule.

Finally, the pure and competitive NOP antagonist properties of Trap-101 have been confirmed in a series of [^{35}S]GTPγS binding experiments performed on CHO_{hNOP} cell membranes. As shown in Figure 2, Trap-101 was inactive per se up to 10 μM while, in the range 3–300 nM, it produced a concentration dependent rightward shift of the concentration–response curve to N/OFQ without modifications of the maximal response to the agonist. Schild analysis of these data yielded a pA_2 value of 8.55. In parallel experiments, (±)J-113397 displayed the same profile (pure and competitive antagonist behavior) but it showed a 3-fold higher pA_2 value (9.08).

5. Conclusions

Investigating the importance of the chirality of J-113397 we identified a structurally related achiral compound, named Trap-101, that behaves as a potent NOP receptor antagonist. This molecule represents the first example of a NOP receptor ligand characterized by a 1,2,3,6-tetrahydro-pyridine nucleus. Trap-101 shows, in the mouse vas deferens assay and in receptor binding and [^{35}S]GTPγS binding experiments on recombinant NOP receptors, a pharmacological profile very close to that of J-113397; however, the preparation of Trap-101 is much easier than that of J-113397 and the yield much higher. A detailed characterization of the in vitro and in vivo pharmacological profile of Trap-101 in comparison with J-113397 is under way in our laboratories. The results of these studies will define the value of Trap-101 as a novel pharmacological tool for investigating the biological functions modulated by the N/OFQ-NOP receptor system.

6. Experimental

6.1. Materials

Reagents and solvents were obtained from commercial suppliers and used without further purification. Solvents used were of either analytical reagent or HPLC grade. Dry DMF was prepared and stored over molecular sieves (11X).

6.2. Chemistry

Melting points (uncorrected) were measured with a Buchi-Tottoli apparatus, and ^{1}H, ^{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. MS analyses were performed on a ESI-Micromass ZMD 2000. Infrared spectra were recorded on a Perkin-Elmer FT-IR Paragon 500 spectrometer. Flash chromatography was carried out on a silica gel (Merck, 230–400 Mesh).

Elemental analyses were performed by the microanalytical laboratory of Dipartimento di Chimica, University of Ferrara.

7. Synthesis

7.1. 3-(1-Cyclooctylmethyl-5-methoxycarbonyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-oxo-2,3-dihydro-benzoimidazole-1-carboxylic acid *tert*-butyl ester (*I*)

A cooled (0 °C) solution of 4-(2-amino-phenylamino)-1-cyclooctylmethyl-1,2,5,6-tetrahydro-pyridine-3- carboxylic acid methyl ester (REF) (2 g, 5,39 mmol) in CH_2Cl_2 (40 mL) was treated with di-*tert*-butyldicarbonate (5.88 g, 26.95 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred at the same temperature for 3 h, and then the solvent was evaporated in vacuo and the residue was purified by flash chromatography

(EtOAc/light petroleum, 1:6) yielding **I** (2.4 g, 89%) as an orange oil. Anal. Calcd for $C_{28}H_{39}N_3O_5$: C, 67.58; H, 7.90; N, 8.44. Found: C, 67.61; H, 7.88; N, 8.43. MS (ESI): $[MH]^+ = 498$. IR (KBr): 1742, 1724, 1647, 1580, 1500 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.83–7.90 (m, 1H); 7.08–7.18 (m, 2H); 6.80–6.90 (m, 1H); 3.47 (s, 3H); 3.45 (AB system, 2H, $J = 15$ Hz); 2.60–3.00 (m, 4H); 2.29 (d, 2H, $J = 7.2$ Hz); 1.20–1.90 (m, 24H). Analytical data are in accord with the literature.

7.2. 1-Cyclooctylmethyl-4-(2-oxo-2,3-dihydro-benzoimidazol-1-yl)- 1,2,5,6-tetrahydro-pyridine-3-carboxylic acid methyl ester (II)

To a solution of **I** (2 g, 4.02 mmol) in dichloromethane (20 mL) at 0 °C, TFA (2.17 mL, 28.14 mmol) was added dropwise. The reaction mixture was kept at rt until the completion (8 h), and then saturated aq $NaHCO_3$ (20 mL) was carefully added. The phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3× 20 mL). The combined organic layers were dried. Evaporation of the solvent gave **II** (1.54 g, 97%) as a yellow amorphous solid (mp 28–30 °C). Anal. calcd for $C_{23}H_{31}N_3O_3$: C, 69.49; H, 7.86; N, 10.57. Found: C, 69.51; H, 7.85; N, 10.56. MS (ESI): $[MH]^+ = 398$. IR (KBr): 3422, 1707, 1487 cm^{-1}. ^1H NMR ($CDCl_3$): δ 9.64 (s, 1 H); 7.05–7.20 (m, 3H); 6.85–7.00 (m, 1H); 3.30–3.60 (s, 3H, superimposed at 3.44); 2.60–2.90 (m, 4H); 2.31 (d, 2H, $J = 7.1$ Hz); 1.20–1.90 (m, 15H).

7.3. 1-Cyclooctylmethyl-4-(3-ethoxycarbonylmethyl-2-oxo-2,3- dihydro- benzoimidazol-1-yl)-1,2,5,6-tetrahydro-pyridine-3-carboxylic acid methyl ester (III)

A solution of **II** (0.4 g, 1.007 mmol) in anhydrous DMF (10 mL) was added dropwise to a stirred suspension of 75% NaH (44 mg, 1.10 mmol) in anhydrous DMF (5 mL) at 0 °C. The reaction mixture was stirred for 30 min at the same temperature, and then a solution of ethyl bromoacetate (0.122 mL, 1.10 mmol) in DMF (5 mL) was added slowly and stirring was continued overnight at rt. Most of the solvent was evaporated and the residue was diluted with ethyl ether (50 mL). The precipitated salts were filtered over a Celite pad, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography (EtOAc/light petroleum/NH_4OH, 1:4:0.1) to give **III** (0.36 g 75%) as a yellow oil. Anal. Calcd for $C_{27}H_{37}N_3O_5$: C, 67.06; H, 7.71; N, 8.69. Found: C, 67.08; H, 7.72; N, 8.70. MS (ESI): $[MH]^+ = 484{,}5$. ^1H NMR ($CDCl_3$): 7.05–7.07 (m, 2H); 6.87–6.91 (m, 2H); 5.29 (s, 1H); 4.63 (s, 2H); 4.20–4.26 (q, 2H, $J = 7$ Hz); 3.42 (s, 3H); 2.27–2.29 (d, 2H, $J = 7$ Hz); 1.49–1.74 (m, br, 18H); 1.24–1.30 (m, 6H). ^{13}C NMR ($CDCl_3$): 167.70; 165.10; 152.41; 138.23; 129.38; 129.14; 127.94; 121.96; 121.82; 108.64; 107.8; 65.02; 61.82; 53.51; 53.13; 51.91; 49.42; 42.30; 35.03; 30.75; 29.68; 27.26; 26.49; 25.63; 14.24.

7.4. C-Cyclooct-1-enyl-methylamine (IV)

A solution of 1-nitromethylcyclooctene (6 g, 35.50 mmol) in anhydrous THF (20 mL) was added dropwise to a suspension of $LiAlH_4$ (4 g, 106.5 mmol) in anhydrous THF (80 mL) cooled at 0 °C. The reaction mixture was stirred at the same temperature for 6 h, after that icey-cooled water was carefully added and the aluminum salts were filtered through Celite pad. The organic fraction was evaporated in vacuo to obtain a yellow oil (4.23 g 86%) **IV**. Anal. Calcd for $C_9H_{17}N$: C, 77.63; H, 12.31; N, 10.06. Found: C, 77.61; H, 12.29; N, 10.08. MS (ESI): $[MH]^+ = 140.3$. IR (film): 3300, 1579 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.44 (t, 1H, $J = 8.1$); 3.16 (s, 2H); 1.3–2.2 (m, 12H).

7.5. 3-[Cyclooct-1-enylmethyl-(2-methoxycarbonyl-ethyl)-amino]- propionic acid methyl ester (V)

A solution of (**IV**) (5 g, 35.97 mmol) in methanol (20 mL) was added dropwise to a solution of methylacrylate (7 mL, 78.01 mmol) in methanol (50 mL). The reaction mixture was stirred at rt for 24 h, the solvent was evaporated in vacuo, and the residues was purified by flash chromatography (EtOAc/light petroleum 1:6) to give the product (**V**) as a light yellow oil (8 g, 72%). Anal. Calcd for $C_{17}H_{29}NO_4$: C, 65.57; H, 9.39; N, 4.50. Found: C, 65.56; H, 9.41; N, 4.47. MS (ESI): $[MH]^+ = 312.4$. ^1H NMR ($CDCl_3$): δ 5.32 (t, 1H, $J = 8.1$ Hz); 3.67 (s, 6H); 3.03 (s, 2H); 2.75 (t, 4H, $J = 7$ Hz); 2.735 (t, 4H, $J = 7$ Hz); 1.20–1.96 (m, 12H).

7.6. 1-Cyclooct-1-enylmethyl-4-oxo-piperidine-3-carboxylic acid methyl ester (VI)

To a solution of **V** (8 g, 24.46 mmol) in toluene (80 mL) cooled at 0 °C was added in one portion ^{t}ButOK (4.3 g 38.34 mmol). The reaction mixture was stirred for 30 min at the same temperature and then for 12 h at rt. One hundred millilitres of water was added, the phases were separated, and the aqueous layer was extracted 3 times with EtOAc. The combined organic phases were dried and concentrated in vacuo. The residue was purified by flash chromatography (EtOAc/light petroleum 1:9) to give **VI** as a orange oil (5.6 g 78%). Anal. Calcd for $C_{16}H_{25}NO_3$: C, 68.79; H, 9.02; N, 5.01. Found: C, 65.56; H, 9.41; N, 4.47. MS (ESI): $[MH]^+ = 280.4$. ^1H NMR ($CDCl_3$): δ 11.87 (s, b, 1H enolic form); 5.30 (t, 1H, $J = 8.1$ Hz); 3.73 (s, 3H); 3.07 (AB system, 2H, $J = 10$ Hz); 2.56 (t, 2H, $J = 7$ Hz); 2.37 (t, 2H, $J = 7$ Hz); 2.19 (d, 2H, $J = 7.3$ Hz); 1.5–1.85 (m, 12H).

7.7. 4-(2-Amino-phenylamino)-1-cyclooct-1-enylmethyl-1,2,5,6-tetrahydro-pyridine-3-carboxylic acid methylester (VII)

A solution of **VI** (3 g, 10.75 mmol) and *o*-phenylenediamine (1.77 g, 16.43 mmol) in benzene (50 mL) was heated at reflux overnight in the presence of AcOH (0.3 mL) and molecular sieves 4 Å (1 g) using a Dean-stark apparatus. The solution was cooled at rt, filtered, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography using as eluent EtOAc/light petroleum/NH_4OH, 1:4:0.3 to give **VII** (3.3 g, 83%). Anal. Calcd for $C_{22}H_{31}N_3O_2$: C, 71.51; H, 8.46; N, 11.37. Found: C, 71.51; H, 8.45; N, 11.37. MS (ESI): $[MH]^+ = 370.3$. ^1H NMR ($CDCl_3$): δ 9.83 (s, 1H); 6.95–7.12 (m, 2H); 6.60–6.80 (m, 2H);

5.36 (t, 1H, $J = 8.1$ Hz); 3.80 (s, 2H); 3.72 (s, 3H); 3.20 (s, 2H); 2.18 (d, 2H, $J = 7.3$ Hz); 2.20–2.50 (m, 4H); 1.20–1.90 (m, 12H).

7.8. 3-(1-Cyclooct-1-enylmethyl-5-methoxycarbonyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-oxo-2,3-dihydro-benzoimidazole-1-carboxylic acid *tert*-butyl ester (VIII)

A solution of **VII** (2 g, 5.42 mmol) in 40 mL DCM cooled at 0 °C was treated with di-*tert*-butyldicarbonate (5.88 g, 26.95 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred at 0 °C for 12 h, the solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography (EtOAc/light petroleum/NH_4OH, 1:9:0.3) to yield **VIII** (2.4 g, 89%) as a orange oil. Anal. Calcd for $C_{28}H_{37}N_3O_5$: C, 67.86; H, 7.52; N, 8.48. Found: C, 67.85; H, 7.54; N, 8.51. MS (ESI): $[MH]^+ = 396.4$ (loss of ^{t}Butoxycarbonyl group). ^{1}H NMR ($CDCl_3$): δ 7.086–7.14 (m, 3H); 6.84 (m, 1H); 5.56 (t, 1H, $J = 8.0$ Hz); 4.41 (s, br, 4H); 3.46 (s, 3H); 3.03 (d, 2H, $J = 3.4$ Hz); 2.23 (d, 2H, $J = 6.4$ Hz); 2.153 (d, 2H, $J = 5.6$ Hz); 1.64 (s, 9H); 1.25–1.54 (m, 10H).

7.9. 1-Cyclooct-1-enylmethyl-4-(3-ethyl-2-oxo-2,3-dihydro- benzoimidazol-1-yl)- 1,2,5,6-tetrahydro-pyridine-3-carboxylic acid methyl ester (IX)

To a solution of **VIII** (2.4g, 4.85 mmol) in DCM (20 mL) cooled at 0 °C was added dropwise TFA (2.6 mL, 33.95 mmol). The reaction mixture was stirred at rt for 8 h, a saturated solution of $NaHCO_3$ (20 mL) was added, the phases were separated, and the aqueous phase was extracted with Et_2O (3× 20 mL). The combined organic phases were dried and evaporated under vacuum to obtain a free amide used in the next step without further purification.

A solution of free amide (1.97g, 4.65 mmol) in 10 mL of anhydrous DMF was added dropwise to a stirred suspension of NaH 75% (0.163g, 5.115 mmol) in DMF (5 mL) at 0 °C under argon atmosphere. The reaction mixture was stirred for 30 min at the same temperature and then a solution of ethyl bromide (0.381 mL, 5.115 mmol) in 5 mL DMF was added; the reaction mixture was kept at rt for 12 h. Most of the solvent was evaporated and the residue was diluted with Et_2O (50 mL), the salts were filtered over a Celite pad, and the solvent was removed under vacuum evaporation. The residue was purified by silica gel chromatography (EtOAc/light petroleum/NH_4OH, 1:4:0.3) yielding **IX** (1.47g, 75%) as a yellow oil. Anal. Calcd for $C_{25}H_{33}N_3O_3$: C, 70.89; H, 7.85; N, 9.92. Found: C, 70.92; H, 7.88; N, 9.89. MS (ESI): $[MH]^+ = 424.5$. ^{1}H NMR ($CDCl_3$): δ 7.00–7.07 (m, 3H); 6.88 (m, 1H); 5.54–5.58 (t, 1H, $J = 8$ Hz); 3.93–395 (q, 2H, $J = 7.2$ Hz); 3.39 (s, 3H); 3.03 (s, 2H); 2.68 (s, br, 6H); 2.24 (t, 2H, $J = 6.4$ Hz); 2.13 (m, 2H); 1.48–1.53 (m, 8H); 1.34 (t, 3H, $J = 7.2$ Hz).

^{13}C NMR ($CDCl_3$): δ 164.93; 152.22; 138.71; 137.75; 129.32; 129.11; 127.87; 127.60; 121.46; 121.22; 108.42; 107.68; 63.93; 52.99; 51.76; 48.54; 35.91; 29.80; 29.68; 28.89; 27.33; 26.54; 26.48; 26.18; 13.6.

7.10. -*cis*-1-(1-Cyclooctylmethyl-3-hydroxymethyl-piperidin-4-yl)-3-ethyl-1,3-dihydro-benzoimidazol-2-one (1)

A solution of (3) (0.03 g, 0.07 mmol) in anhydrous THF (2 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (5.85 mg, 0.154 mmol) in anhydrous THF (5 mL). After stirring for 20 min at the same temperature, water (3 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and preparative HPLC using a linear gradient 20–100% CH_3CN in 30 min gave racemic (1) (25.2 mg, 90%) as a yellow oil. Anal. Calcd for $C_{24}H_{37}N_3O_2$: C, 72.14; H, 9.33; N, 10.51. Found: C, 72.15; H, 9.38; N, 10.53. MS (ESI): $[MH]^+ = 400.4$.

^{1}H NMR ($CDCl_3$): δ 7.85–7.88 (m, 1H); 7.07–7.09 (m, 3H); 6.98–7.00 (m, 1H); 4.68–4.74 (dt, 1H $J = 14$ Hz; $J = 5$ Hz); 3.91–3.97 (m, 4H); 3.21–3.31 (dq, 2H, $J = 11.6$ Hz; $J = 4$ Hz); 3.14–3.17 (d, 2H, $J = 11.6$ Hz); 2.59–2.62 (dt, 1H, $J = 11.6$ Hz; $J = 2$ Hz); 2.14–2.18 (m, 3H); 2.03 (s, dr, 1H); 1.49–1.70 (m, 14H); 1.34 (t, 3H, $J = 7$ Hz).

^{13}C NMR ($CDCl_3$): 154.04; 129.53; 128.81; 121.22; 120.85; 110.97; 107,21; 66.01; 65.13; 59.77; 54.08; 54.04; 39.92; 35.92; 34.40; 30.77; 27.15; 26.90; 26.70; 26.38; 25.74; 25.02; 13.55.

7.11. (±)-*trans*-1-Cyclooctylmethyl-4-(3-ethyl-2-oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidine-3-carboxylic acid methyl ester (2)

A solution of (3) (0.5g, 1.17 mmol) in anhydrous methanol was added dropwise to a solution of MeONa, freshly prepared dissolving sodium (0.19 g, 8.26 mmol) in anhydrous methanol (5 mL) at 0 °C. The reaction mixture was stirred at rt for 60 h, then adsorbed on silica gel (1 g) and subjected to flash chromatography (EtOAc/light petroleum/NH_4OH, 1:4:0.1) to give (2) (0.46 g, 92%) as a white solid (mp 68–70 °C). Anal. Calcd for $C_{25}H_{37}N_3O_3$: C, 70.23; H, 8.72; N, 9.83. Found: C, 70.19; H, 8.73; N, 8.95. MS (ESI): $[MH]^+ = 428$. IR (KBr): 1745, 1698, 1616, 1490 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.00–7.20 (m, 4H); 4.39 (dt, 1H, $J = 12.2$, 5 Hz); 3.91 (q, 2H, $J = 7$ Hz); 3.68 (m, 1H); 3.43 (s, 3H); 3.10–3.25 (m, 2H); 2.90–3.08 (m, 1H); 2.56 (dq, 1H, $J = 12.5$, 3.9 Hz); 2.08–2.30 (m, 5H); 1.40–1.80 (m, 15H); 1.33 (t, 3H, $J = 7$ Hz). Analytical data are in accord with the literature.

7.12. (±)-*cis*-1-Cyclooctylmethyl-4-(3-ethyl-2-oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidine-3-carboxylic acid methyl ester (3)

A solution of (5) (0.3 g, 0.70 mmol) in methanol (15 mL), was hydrogenated in bomb at 60 atmosphere for 24 h in the presence of Pd/C 10% (0.1 g). Filtration of the catalyst through a Celite pad, solvent evaporation, and flash chromatography (EtOAc/light petroleum/NH_4OH, 1:4:0.1) of the residue gave (3) (0.21 g 70%) as a colorless oil. Anal Calcd for $C_{25}H_{37}N_3O_3$: C, 70.23; H, 8.72; N, 9.83. Found: C, 70.26; H, 8.70; N, 9.81. MS (ESI): $[MH]^+ = 428$. IR (film): 1745, 1698, 1616, 1490 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.50–7.70 (m, 1H); 6.95–7.10 (m,

3H); 4.39 (dt, 1H, $J = 13.3$, 4 Hz); 3.93 (dq, 2H, $J = 7$, 2.4 Hz); 3.53 (s, 3H); 3.20–3.48 (m, 2H); 3.00–3.15 (m, 1H); 2.37 (dd, 1H, $J = 11.6$, 3 Hz); 1.90–2.30 (m, 5H); 1.40–1.80 (m, 15H); 1.33 (t, 3H, $J = 7$ Hz). Analytical data are in accord with the literature.

7.13. [1-Cyclooctylmethyl-4-(3-ethyl-2,3-dihydro-benzoimidazol-1-yl)-piperidin-3-yl]-methanol (4)

A solution of (2) (0.5 g, 1.17 mmol) in anhydrous THF (3 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (5.02 mg, 0.132 mmol) in anhydrous THF (8 mL). After stirring for 50 min at the same temperature, water (5 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent gave (4) (383 mg 85%) without further purification as a yellow oil. Anal. Calcd for $C_{24}H_{39}N_3O$: C, 74.76; H, 10.19; N, 10.90. Found: C, 74.76; H, 10.21; N, 10.92. MS (ESI): $[MH]^+ = 386.6$. ^1H NMR ($CDCl_3$): δ 7.85–7.88 (m, 1H); 7.07–7.09 (m, 3H); 6.98–7.00 (m, 1H); 5.41 (s, 2H); 4.68–4.74 (dt, 1H $J = 14$ Hz; $J = 5$ Hz); 3.91–3.97 (m, 4H); 1.13 (t, 3H, $J = 7$ Hz); 3.21–3.31 (dq, 2H, $J = 11.6$ Hz; $J = 4$ Hz); 3.14–3.17 (d, 2H, $J = 11.6$ Hz); 2.59–2.62 (dt, 1H, $J = 11.6$ Hz; $J = 2$ Hz); 2.14–2.18 (m, 3H); 2.03 (s, br, 1H); 1.30–1.60 (m, 14H).

^{13}C NMR ($CDCl_3$): 129.53; 128.81; 121.22; 120.85; 110.97; 107,21; 68.02; 66.01; 65.13; 59.77; 54.08; 54.04; 39.92; 35.92; 34.40; 30.77; 27.15; 26.90; 26.70; 26.38; 25.74; 25.02; 13.55.

7.14. 1-Cyclooctylmethyl-4-(3-ethyl-2-oxo-2,3-dihydro-benzoimidazol-1-yl)-1,2, 5,6-tetrahydro-pyridine-3-carboxylic acid methyl ester (5)

A solution of **II** (1.5 g, 3.78 mmol) in anhydrous DMF was added dropwise to a stirred suspension of 75% NaH (.12 g, 3.78 mmol) in anhydrous DMF (5 mL) at 0 °C. The reaction mixture was stirred for 30 min at the same temperature, then a solution of ethyl bromide (0.31 mL, 4.16 mmol) in DMF (5 mL) was added slowly and stirring was continued overnight at rt. Most of the solvent was evaporated and the residue was diluted with ethyl ether (50 mL). The precipitated salts were filtered over a Celite pad, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography (EtOAc/light petroleum/NH_4OH, 1:4:0.1) to give (5) (1.2 g 75%) as a yellow oil. Anal Calcd for $C_{25}H_{35}N_3O_3$: C, 70.56; H, 8.29; N, 9.87. Found: C, 70.58; H, 8.28; N, 9.86. MS (ESI): $[MH]^+ = 426$. IR (film): 1714, 1647, 1616, 1493 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.05–7.10 (m, 3H); 6.80–7.00 (m, 1H); 3.88 (q, 2H, $J = 7.2$ Hz); 3.30–3.50 (m, 2H, superimposed to s, 3H a 3.34); 2.50–2.80 (m, 4H); 2.22 (d, 2H, $J = 7.2$ Hz); 1.40–1.80 (m, 15H); 1.28 (t, 3H, $J = 7.2$ Hz).

7.15. 1-(1-Cyclooctylmethyl-5-hydroxymethyl-1,2,3,6-tetrahydro-pyridin-4-yl)-1, 3-dihydro-benzoimidazol-2-one (6)

A solution of **II** (1 g, 2.52 mmol) in anhydrous THF (15 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (0.98 g, 2.57 mmol) in anhydrous THF (20 mL). After stirring for 20 min at the same temperature, water (10 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and flash chromatography purification (EtOAc/light petroleum/NH_4OH, 2:1:0.1) gave (6) (0.65 g 70%) as a yellow oil. Anal. Calcd for $C_{22}H_{31}N_3O_2$: C, 71.51; H, 8.46; N, 11.37. Found: C, 71.47; H, 8.48; N, 11.35. MS (ESI): $[MH]^+ = 370,6$. ^1H NMR ($CDCl_3$): 7.06–7.10 (m, 3H); 6.95 (m, 1H); 4.16 (d, 1H, $J = 16$ Hz); 3.59 (m, 2H); 2.5–3.1 (m, br, 6H); 2.25–2.29 (d, 2H, $J = 16$ Hz); 2.02 (m, 2H); 1.53–1.75 (m, br, 12H); 1.24–1.28 (m, 2H).

7.16. 3-(1-Cyclooctylmethyl-5-hydroxymethyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-oxo-2,3-dihydro-benzoimidazole-1-carboxylic acid *tert*-butyl ester (7)

A solution of **I** (30 mg, 0.06 mmol) in anhydrous THF (1 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (5.02 mg, 0.132 mmol) in anhydrous THF (5 mL). After stirring for 20 min at the same temperature, water (3 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and flash chromatography purification (EtOAc/light petroleum/NH_4OH, 3:1:0.3) gave (7) (19 mg 67%) as a colorless oil. Anal. Calcd for $C_{27}H_{39}N_3O_4$: C, 69.05; H, 8.37; N, 8.95. Found: C, 69.02; H, 8.39; N, 8.94. MS (ESI): $[MH]^+ = 470.6$. ($[MH]^+ = 370.6$ *tert*-butoxycarbonyl moiety). ^1H NMR ($CDCl_3$): 7.1 (m, 3H); 6.9 (m, 1H); 6.05 (s, br, 2H); 5.8 (s, br, 2H); 4.05 (m, 1H); 3.69–3.75 (t, 4H, $J = 6.6$ Hz); 2.79 (m, 2H); 2.28 (m, 2H); 1.99 (s, 9H); 1.51–1.86 (m, 12H).

7.17. 1-(1-Cyclooctylmethyl-5-hydroxymethyl-1,2,3,6-tetrahydro-pyridin-4-yl)-3- (2-hydroxy-ethyl)-1,3-dihydro-benzoimidazol-2-one (8)

A solution of **III** (0.1 g, 0.21 mmol) in anhydrous THF (5 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (17.3 mg, 0.455 mmol) in anhydrous THF (7 mL). After stirring for 20 min at the same temperature, water (5 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and flash chromatography purification (EtOAc/light petroleum/NH_4OH, 4:1:0.3) gave (8) (62 mg 72%) as a colourless oil. Anal. Calcd for $C_{24}H_{35}N_3O_3$: C, 69.70; H, 8.53; N, 10.16. Found: C, 69.67; H, 8.50; N, 8.13. MS (ESI): $[MH]^+ = 414.2$. ^1H NMR ($CDCl_3$): 7.04–7.10 (m, 3H); 6.88–6.89 (m, 1H); 3.99–4.02 (m, 3H); 3.87–3.89 (m, 2H); 3.58–3.61 (d, 1H, $J = 12$ Hz); 3.40–3.45 (d, br, 1H, $J = 16.8$ Hz); 3.02–3.07 (d, 1H, $J = 16.8$ Hz); 2.70–2.76 (m, 1H); 2.58–2.63 (m, 1H); 2.43–2.47 (d, br, 1H, $J = 17$ Hz); 2.26–2.31 (br, 1H); 2.22–2.24 (d, 2H, $J = 7.6$ Hz); 1.56–1.94 (m, br, 6H); 1.45–1.55 (m, br, 7H); 1.20–1.23 (m, 2H).

^{13}C NMR ($CDCl_3$): 173.47; 154.30; 136.75; 129.78; 128.73; 125.64; 122.07; 121.74; 109.33; 108.56; 65.47; 60.22; 59.61; 54.53; 50.04; 44.26; 34.76; 30.76; 30.68; 27.83; 27.11; 26.36; 25.50; 22.62.

7.18. 1-(1-Cyclooct-1-enylmethyl-5-hydroxymethyl-1,2,3,6-tetrahydro-pyridin-4-yl)-3-ethyl-1,3-dihydro-benzoimidazol-2-one (9)

A solution of **IX** (1 g, 2.36 mmol) in anhydrous THF (10 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (197 mg, 5.192 mmol) in anhydrous THF (20 mL). After stirring for 20 min at the same temperature, water (10 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and purification by flash chromatography (EtOAc/light petroleum/NH_4OH, 1:2:0.3) gave (9) (0.91g 97%) as a white oil. Anal. Calcd for $C_{24}H_{33}N_3O_2$: C, 72.88; H, 8.41; N, 10.62. Found: C, 72.90; H, 8.44; N, 10.65. MS (ESI): $[MH]^+ = 396.5$. ^1H NMR ($CDCl_3$): δ 7.03–7.13 (m, 3H); 6.94–6.95 (m, 1H); 5.56 (t, 1H); 4.06 (d, 1H, $J = 12$ Hz); 3.96 (q, 2H, $J = 12$ Hz); 3.58 (d, 1H, $J = 12$ Hz); 3.12–3.52 (2d, 2H, $J = 16.4$ Hz); 3.02 (s, 2H); 2.65–2.74 (m, 2H); 2.58 (d, 1H); 2.25–2.26 (m, 2H); 2.13 (m, 2H); 1.47–1.54 (m, 10H); 1.36 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): 153.69; 137.87; 136.83; 129.27; 128.88; 127.81; 126.03; 121.92; 121.53; 109.54; 108.03; 64.16; 60.05; 54.65; 49.33; 36.33; 29.84; 28.85; 27.84; 27.27; 26.57; 26.48; 26.20; 13.64.

7.19. (±)-1-(1-Cyclooctylmethyl-3-hydroxymethyl-piperidin-4-yl)-3-ethyl-1,3-dihydro-benzoimidazol-2-one. (±)-J-113397

A solution of (2) (1 g, 2.34 mmol) in anhydrous THF (15 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (0.19 g, 5.01 mmol) in anhydrous THF (20 mL). After stirring for 20 min at the same temperature, water (10 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and flash chromatography purification (EtOAc/light petroleum/NH_4OH, 1:2:0.1) gave racemic J-113397 (0.89g, 95%) as a white solid (mp 93–95 °C). Analytical data are in accord with the literature (REF). Anal. Calcd for $C_{24}H_{37}N_3O_2$: C, 72.14; H, 9.33; N, 10.51. Found: C, 72.18; H, 9.30; N, 10.51. MS (ESI): $[MH]^+ = 400$. IR (KBr): 3435, 1686, 1491 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.31–7.33 (d, 1H, $J = 7.2$ Hz); 7.03–7.11 (m, 3H); 4.35–4.40 (t, br, 1H, $J = 11.6$ Hz); 4.09–4.14 (q, 1H, $J = 7.2$ Hz); 3.90–4.01 (m, 2H); 3.33 (s, 2H); 2.98–3.02 (t, 3H, $J = 6.4$ Hz); 2.58–2.62 (dq, 1H, $J = 8.2$ Hz, $J = 4$ Hz,); 2.17–2.29 (m, 2H); 2.13–2.15 (d, 2H, $J = 6.8$ Hz); 2.01–2.06 (m, 3H); 1.88 (dd, 1H); 1.47–1.74 (m, 12H); 1.32–1.35 (t, 3H, $J = 7.2$ Hz).

^{13}C NMR ($CDCl_3$): 154.67; 129.30; 128.09; 121.22; 110.32; 108.02; 66.14; 61.95; 60.48; 56.53; 53.65; 51.80; 41.07; 36.15; 34.92; 30.97; 30.92; 28.80; 27.22; 27.20; 26.54; 25.67; 14.26; 13.64. Analytical data are in line with the literature.

7.20. 1-(1-Cyclooctylmethyl-5-hydroxymethyl-1,2,3,6-tetrahydro-pyridin-4-yl)-3-ethyl-1,3-dihydro-benzoimidazol-2-one. (Trap-101)

A solution of (5) (0.5 g, 1.17 mmol) in anhydrous THF (15 mL) was added dropwise to a cooled (0 °C) slurry of $LiAlH_4$ (0.98 g, 2.57 mmol) in anhydrous THF (20 mL). After stirring for 20 min at the same temperature, water (10 mL) was slowly added, and the precipitated aluminum salts were filtered through a Celite pad. Evaporation of the solvent and flash chromatography purification (EtOAc/light petroleum/NH_4OH, 1:2:0.1) gave (Trap-101) as a slurry yellow oil in quantitative yield. Anal. Calcd for $C_{24}H_{35}N_3O_2$: C, 72.51; H, 8.87; N, 10.57. Found: C, 72.49; H, 8.90; N, 10.55. MS (ESI): $[MH]^+ = 398.5$. ^1H NMR ($CDCl_3$): 7.04–7.13 (m, 3H); 6.94–6.96 (m, 1H); 4.05–4.08 (d, 1H, $J = 11.6$ Hz); 3.95–3.98 (m, 2H); 3.72–3.75 (m, 3H); 3.56 (m, 2H); 3.06–3.10 (dt, 1H, $J = 16.5$ Hz, $J = 2.5$ Hz); 2.74–2.79 (m, 1H); 2.55–2.59 (d, br, 1H); 2.65–2.70 (m, 1H); 2.25–2.28 (m, 2H); 1.83–1.84 (m, 2H); 1.48–1.75 (m, 12H); 1.34–1.36 (t, 3H, $J = 7$ Hz).

^{13}C NMR ($CDCl_3$): 153.71; 136.70; 129.28; 128.88; 125.94; 121.93; 121.52; 109.58; 108.04; 68.04; 65.42; 60.06; 54.82; 50.14; 36.35; 34.94; 30.85; 30.75; 27.89; 27.21; 26.49; 25.66; 25.62; 13.65.

8. Molecular modeling procedures

Molecular modeling studies were performed on an Octane R12000 Silicon Graphics workstation. Molecular structure was generated using SYBYL (Version 6.9) supplied by Tripos Associates, St. Louis, Missouri, USA. Three-dimensional model of the molecules was generated starting from the crystal structure of J-113397.[39] as deposited at Cambridge Crystallographic Data Centre (CCDC 151492). Assuming physiological conditions, all the compounds were considered protonated on the basic nitrogen atom of piperidine system. For each compound, the piperidine ring was maintained in a chair conformation, energetically preferred compared to the boat conformation, as found in the starting crystal structure of molecule J-113397.[39] The geometry was optimized using the Tripos force field without including electrostatic terms, applying the conjugate gradient method. Semiempirical molecular orbital calculations were done using the AM1 Hamiltonian[43] as implemented in MOPAC. A conformational analysis was carried out on 3 routable bonds using the Monte Carlo options implemented in MacroModel 8.1.[53] The maximum number of attempts was 5000 and the energy cut-off was set at 100 kcal/mol above the estimated total energy of the molecule. The low energy conformers produced by the random conformational search were fully reoptimized with the semiempirical quantum mechanics calculations AM1.[43] Resulting structures were superimposed on the piperidine ring and on the centroids of the cyclooctyl and benzimidazolidinone system of J-113397, applying the superimposition procedure as implemented in SYBYL.

9. Pharmacological studies

9.1. Electrically stimulated mouse vas deferens

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.[41] 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 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 or J-113397 derivatives were performed (0.5 log unit steps). When tested as antagonists the compounds were added to the Krebs solution 15 min before performing the concentration–response curve to N/OFQ.

9.2. Receptor binding and stimulation of GTPγS binding to human recombinant receptors

The binding of Trap-101 to recombinant human NOP, MOP, DOP, and KOP expressed in Chinese hamster ovary cells was assessed by the displacement of either a fixed concentration of [^{3}H]N/OFQ (for NOP) or [^{3}H]diprenorphine (for MOP, DOP, and KOP) and increasing concentrations of Trap-101 as previously described.[54] Upstream functional antagonism of Trap-101 was assessed in a standard GTPγ^{35}S binding protocol[54] using 3, 30, and 300 nM Trap-101 against N/OFQ.

The pharmacological terminology adopted in this study is in line with IUPHAR recommendations:[55] the agonist potencies are given as pEC_{50} = the negative logarithm to 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: $pA_2 = \log((CR-1)/[\text{antagonist}])$ assuming a slope value equal to unity. In receptor binding studies, the concentration of displacer producing 50% displacement was corrected for the competing mass of radiolabel to yield K_i according to Cheng and Prusoff.[56]

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References and notes

1. 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. *The IUPHAR Compendium of Receptor Characterization and Classification*, 2nd ed.; IUPHAR Media Ltd: London, 2000; pp 321–333.
2. Mogil, J. S.; Pasternak, G. W. *Pharmacol. Rev.* **2001**, *53*, 381–415.
3. Calo, G.; Guerrini, R.; Rizzi, A.; Salvadori, S.; Regoli, D. *Br. J. Pharmacol.* **2000**, *129*, 1261–1283.
4. 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*, 792–794.
5. 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*, 532–535.
6. Wise, A.; Jupe, S. C.; Rees, S. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 43–66.
7. Kawamoto, H.; Ozaki, S.; Itoh, Y.; Miyaji, M.; Arai, S.; Nakashima, H.; Kato, T.; Ohta, H.; Iwasawa, Y. *J. Med. Chem.* **1999**, *42*, 5061–5063.
8. Hashiba, E.; Harrison, C.; Galo, G.; Guerrini, R.; Rowbotham, D. J.; Smith, G.; Lambert, D. G. *Naunyn Schmiedebergs Arch. Pharmacol.* **2001**, *363*, 28–33.
9. Ozaki, S.; Kawamoto, H.; Itoh, Y.; Miyaji, M.; Azuma, T.; Ichikawa, D.; Nambu, H.; Iguchi, T.; Iwasawa, Y.; Ohta, H. *Eur. J. Pharmacol.* **2000**, *402*, 45–53.
10. Hashiba, E.; Lambert, D. G.; Jenck, F.; Wichmann, J.; Smith, G. *Life Sci.* **2002**, *70*, 1719–1725.
11. Bigoni, R.; Calo, G.; Rizzi, A.; Guerrini, R.; De Risi, C.; Hashimoto, Y.; Hashiba, E.; Lambert, D. G.; Regoli, D. *Naunyn Schmiedebergs Arch. Pharmacol.* **2000**, *361*, 565–568.
12. Ho, M.; McKnight, A. T. *Br. J. Pharmacol.* **2000**, *131*, 159P.
13. Corboz, M. R.; Rivelli, M. A.; Egan, R. W.; Tulshian, D.; Matasi, J.; Fawzi, A. B.; Benbow, L.; Smith-Torhan, A.; Zhang, H.; Hey, J. A. *Eur. J. Pharmacol.* **2000**, *402*, 171–179.
14. Tada, H.; Nakagawa, K.; Yamamura, T.; Takahashi, T. *Eur. J. Pharmacol.* **2002**, *454*, 53–58.
15. Olianas, M. C.; Onali, P. *Br. J. Pharmacol.* **2002**, *135*, 233–238.
16. Yamada, S.; Kusaka, T.; Urayama, A.; Kimura, R.; Watanabe, Y. *Br. J. Pharmacol.* **2003**, *139*, 1462–1468.
17. Ichikawa, D.; Ozaki, S.; Azuma, T.; Nambu, H.; Kawamoto, H.; Iwasawa, Y.; Takeshima, H.; Ohta, H. *Neuroreport* **2001**, *12*, 1757–1761.
18. Rominger, A.; Forster, S.; Zentner, J.; Dooley, D. J.; McKnight, A. T.; Feuerstein, T. J.; Jackisch, R.; Vlaskovska, M. *Br. J. Pharmacol.* **2002**, *135*, 800–806.
19. Mela, F.; Marti, M.; Ulazzi, L.; Vaccari, E.; Zucchini, S.; Trapella, C.; Salvadori, S.; Beani, L.; Bianchi, C.; Morari, M. *Eur. J. Neurosci.* **2004**, *19*, 1317–1324.
20. Marti, M.; Stocchi, S.; Paganini, F.; Mela, F.; De Risi, C.; Calo', G.; Guerrini, R.; Barnes, T. A.; Lambert, D. G.; Beani, L.; Bianchi, C.; Morari, M. *Br. J. Pharmacol.* **2003**, *138*, 91–98.
21. Luo, C.; Kumamoto, E.; Furue, H.; Chen, J.; Yoshimura, M. *Neuroscience* **2002**, *109*, 349–358.
22. Jennings, E. A. *Neuroreport* **2001**, *12*, 645–648.
23. Vaughan, C. W.; Connor, M.; Jennings, E. A.; Marinelli, S.; Allen, R. G.; Christie, M. J. *J. Physiol.* **2001**, *534*, 849–859.
24. Chiou, L. C.; Fan, S. H. *Neuropharmacology* **2002**, *42*, 987–992.
25. Ueda, H.; Inoue, M.; Takeshima, H.; Iwasawa, Y. *J. Neurosci.* **2000**, *20*, 7640–7647.

26. Ko, M. C.; Naughton, N. N.; Traynor, J. R.; Song, M. S.; Woods, J. H.; Rice, K. C.; McKnight, A. T. *Br. J. Pharmacol.* **2002**, *135*, 943–950.
27. Corboz, M. R.; Fernandez, X.; Egan, R. W.; Hey, J. A. *Life Sci.* **2001**, *69*, 1203–1211.
28. Bolser, D. C.; McLeod, R. L.; Tulshian, D. B.; Hey, J. A. *Eur. J. Pharmacol.* **2001**, *430*, 107–111.
29. McLeod, R. L.; Parra, L. E.; Mutter, J. C.; Erickson, C. H.; Carey, G. J.; Tulshian, D. B.; Fawzi, A. B.; Smith-Torhan, A.; Egan, R. W.; Cuss, F. M.; Hey, J. A. *Br. J. Pharmacol.* **2001**, *132*, 1175–1178.
30. Ishihara, S.; Minowa, S.; Tsuchiya, S.; Horie, S.; Watanabe, K.; Murayama, T. *Eur. J. Pharmacol.* **2002**, *441*, 105–114.
31. Yamamoto, T.; Sakashita, Y.; Nozaki-Taguchi, N. *Neuroreport* **2001**, *12*, 1323–1327.
32. Rizzi, A.; Marzola, G.; Zucchini, S.; Trapella, C.; Zeilhofer, H. U.; Bellasio, S.; Bertorelli, R.; Salvadori, S.; Regoli, D.; Calo, G. Endogenous nociceptin/orphanin FQ (N/OFQ) signaling produces antinociceptive effects in the mouse formalin test: pharmacological and genetic evidences. *EPHAR meeting 2004*: Porto, Portugal, 2004.
33. Redrobe, J. P.; Calo, G.; Regoli, D.; Quirion, R. *Naunyn Schmiedeberg's Arch. Pharmacol.* **2002**, *365*, 164–167.
34. Gavioli, E. C.; Marzola, G.; Guerrini, R.; Bertorelli, R.; Zucchini, S.; De Lima, T. C.; Rae, G. A.; Salvadori, S.; Regoli, D.; Calo, G. *Eur. J. Neurosci.* **2003**, *17*, 1987–1990.
35. Bregola, G.; Zucchini, S.; Rodi, D.; Binaschi, A.; D'Addario, C.; Landuzzi, D.; Reinscheid, R.; Candeletti, S.; Romualdi, P.; Simonato, M. *J. Neurosci.* **2002**, *22*, 10030–10038.
36. Lutfy, K.; Eitan, S.; Bryant, C. D.; Yang, Y. C.; Saliminejad, N.; Walwyn, W.; Kieffer, B. L.; Takeshima, H.; Carroll, F. I.; Maidment, N. T.; Evans, C. J. *J. Neurosci.* **2003**, *23*, 10331–10337.
37. Marti, M.; Mela, F.; Veronesi, C.; Guerrini, R.; Salvadori, S.; Federici, M.; Mercuri, N. B.; Rizzi, A.; Franchi, G.; Beani, L.; Bianchi, C.; Morari, M. Blockade of nociceptin/orphanin FQ receptor signalling in rat substantia nigra pars reticulata stimulates nigrostriatal dopaminergic transmission and motor behaviour. *J. Neurosci.* **2004**, *24*, 6659–6666.
38. Marti, M.; Mela, F.; Trapella, C.; Bianchi, C.; Morari, M. Blockade of nociceptin/orphanin FQ transmission attenuates hypokinesia in hemiparkinsonian rats. *Eighth International Congress of Parkinson's Disease and Movement Disorders*: 14–17 June, Rome, Italy, 2004; p P564.
39. Kawamoto, H.; Nakashima, H.; Kato, T.; Arai, S.; Kamata, K.; Iwasawa, Y. *Tetrahedron* **2001**, *57*, 981–986.
40. De Risi, C.; Piero Pollini, G.; Trapella, C.; Peretto, I.; Ronzoni, S.; Giardina, G. A. *Bioorg. Med. Chem.* **2001**, *9*, 1871–1877.
41. Calo, G.; Rizzi, A.; Bogoni, G.; Neugebauer, V.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. *Eur. J. Pharmacol.* **1996**, *311*, R3–R5.
42. Tamura, R.; Sato, M.; Oda, D. *J. Org. Chem.* **1986**, *51*, 4368–4375.
43. Dewar, M. J. S. E.; Zoebish, G.; Healy, E. F. *J. Am. Chem. Soc.* **1985**, *107*, 3902–3909.
44. Broer, B. M.; Gurrath, M.; Holtje, H. D. *J. Comput. Aided Mol. Des.* **2003**, *17*, 739–754.
45. Calo, G.; Rizzi, A.; Bigoni, R.; Guerrini, R.; Salvadori, S.; Regoli, D. *Clin. Exp. Pharmacol. Physiol.* **2002**, *29*, 223–228.
46. Rover, S.; Adam, G.; Cesura, A. M.; Galley, G.; Jenck, F.; Monsma, F. J., Jr.; Wichmann, J.; Dautzenberg, F. M. *J. Med. Chem.* **2000**, *43*, 1329–1338.
47. Wichmann, J.; Adam, G.; Rover, S.; Hennig, M.; Scalone, M.; Cesura, A. M.; Dautzenberg, F. M.; Jenck, F. *Eur. J. Med. Chem.* **2000**, *35*, 839–851.
48. Jenck, F.; Wichmann, J.; Dautzenberg, F. M.; Moreau, J. L.; Ouagazzal, A. M.; Martin, J. R.; Lundstrom, K.; Cesura, A. M.; Poli, S. M.; Roever, S.; Kolczewski, S.; Adam, G.; Kilpatrick, G. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4938–4943.
49. Zaratin, P. F.; Petrone, G.; Sbacchi, M.; Garnier, M.; Fossati, C.; Petrillo, P.; Ronzoni, S.; Giardina, G. A.; Scheideler, M. A. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 454–461.
50. Topham, C. M.; Mouledous, L.; Poda, G.; Maigret, B.; Meunier, J. C. *Protein Eng.* **1998**, *11*, 1163–1179.
51. Thomsen, C.; Hohlweg, R. *Br. J. Pharmacol.* **2000**, *131*, 903–908.
52. Zaveri, N. T.; Jiang, F.; Olsen, C. M.; Deschamps, J. R.; Parrish, D.; Polgar, W.; Toll, L. *J. Med. Chem.* **2004**, *47*, 2973–2976.
53. Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liscamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440–450.
54. Kitayama, M.; Barnes, T. A.; Carra', G.; McDonald, J.; Calo', G.; Guerrini, R.; Rowbotham, D. J.; Smith, G.; Lambert, D. G. *Naunyn-Schmiedebergs Arch. Pharmacol.* **2003**, *368*, 528–537.
55. Neubig, R. R.; Spedding, M.; Kenakin, T.; Christopoulos, A. *Pharmacol. Rev.* **2003**, *55*, 597–606.
56. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.