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Pharmacological characterization of the nociceptin/orphanin FQ receptor non peptide antagonist Compound 24

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ABSTRACT

Compound 24, 1-benzyl-N-{3-[spiroisobenzofuran-1(3H),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide was recently identified as a nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) ligand. In this study, the *in vitro* and *in vivo* pharmacological profiles of Compound 24 were investigated. *In vitro* studies were performed measuring receptor and [^{35}S]GTPγS binding and calcium mobilization in cells expressing the recombinant NOP receptor as well as using N/OFQ sensitive tissues. *In vivo* studies were conducted using the tail withdrawal assay in mice. Compound 24 produced a concentration-dependent displacement of [^{3}H]N/OFQ binding to CHO_{hNOP} cell membranes showing high affinity (pK_i 9.62) and selectivity (1000 fold) over classical opioid receptors. Compound 24 antagonized with high potency the following *in vitro* effects of N/OFQ: stimulation of [^{35}S]GTPγS binding in CHO_{hNOP} cell membranes (pA_2 9.98), calcium mobilization in CHO_{hNOP} cells expressing the $G\alpha_{qi5}$ chimeric protein (pK_B 8.73), inhibition of electrically evoked twitches in the mouse (pA_2 8.44) and rat (pK_B 8.28) vas deferens, and in the guinea pig ileum (pK_B 9.12). In electrically stimulated tissues, Compound 24 up to 1 μM did not modify the effects of classical opioid receptor agonists. Finally *in vivo*, in the mouse tail withdrawal assay, Compound 24 at 10 mg/kg antagonized the pronociceptive and antinociceptive effects of 1 nmol N/OFQ given supraspinally and spinally, respectively. Under the same experimental conditions Compound 24 did not affect the antinociceptive action of 3 nmol endomorphin-1 injected intrathecally. The present study demonstrated that Compound 24 is a pure, competitive, and highly potent non-peptide NOP receptor selective antagonist.

1. Introduction

Nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995) modulates several different biological functions via selective activation of the N/OFQ peptide (NOP) receptor (Lambert, 2008). There are numerous studies describing the central and peripheral actions of N/OFQ and selective NOP agonists. In contrast, relatively few studies are available regarding the effects of selective NOP receptor antagonists. This is mainly due to the fact that only few such molecules are described in the literature (Chiou et al., 2007; Lambert, 2008). Moreover not all the described NOP receptor antagonists are commercially available. Nevertheless, there is convincing evidence that blockade of NOP receptors may be beneficial in some conditions or pathological states. For instance, peptide ([Nphe^1]N/OFQ(1-13)-NH_2 (Calo et al., 2000) and UFP-101 (Calo et al., 2002b)) and non peptide (J-113397 (Ozaki et al., 2000) and SB-612111 (Zaratin et al., 2004)) NOP antagonists were demonstrated to evoke antidepressant like actions in rodents (Gavioli and Calo, 2006; Gavioli et al., 2003, 2004; Redrobe et al., 2002; Rizzi et al., 2007). Interestingly and corroborating antagonist studies $NOP^{-/-}$ mice displayed an antidepressant-like phenotype (Gavioli et al., 2003). Moreover, in a rather elegant series of studies, it has been demonstrated that the endogenous N/OFQ–NOP receptor signalling inhibits motor behaviour and that NOP receptor antagonists (UFP-101, J-113397, and Trap-101 (Trapella et al., 2006)) produce beneficial effects in rodent models of Parkinson's disease (Marti et al., 2004a,b, 2005, 2007; Viaro et al., 2008). This indication has also been confirmed in non-human primates (Viaro et al., 2008; Visanji et al., 2008). Very recent findings indicated that plasma N/OFQ levels in sepsis were higher in patients who died within 30 days (Williams et al., 2008) and this parallels the preclinical observation that the NOP receptor antagonist UFP-101

Abbreviations: N/OFQ, Nociceptin/orphanin FQ; NOP, N/OFQ peptide receptor; Compound 24, 1-benzyl-N-{3-[spiroisobenzofuran-1(3H),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide; CHO, Chinese hamster ovary; DPN, diprenorphine; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; BSA, bovine serum albumin; HBSS, Hank's Balanced Salt Solution; encapsin, hydroxypropyl-beta-cyclodextrin: AUC, area under the curve.

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reduces animal mortality in a rat model of sepsis (Carvalho et al., 2008). To be fully validated and firmly attributed to the NOP receptor antagonist class of drugs, these emerging indications should be confirmed in future studies using several chemically unrelated molecules.

A novel NOP receptor non-peptide antagonist, 1-benzyl-N-{3-[spiroisobenzofuran-1(3H),4′-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide, has been recently identified by Banyu investigators and named Compound 24 (Goto et al., 2006). The synthesis of this novel ligand is relatively easy and the overall yield relatively high (25% in our laboratory, 31% in Goto et al. (2006)). This is particularly true when compared to the synthesis of other non peptide NOP antagonists such as J-113397 and SB-612111 whose synthesis is very difficult and of low overall yield (≈1% for both molecules in our laboratories, C. Trapella personal communication).

Thus, the aim of the present study was the synthesis and detailed investigation of the pharmacological profile of Compound 24. The novel ligand was investigated *in vitro* in receptor binding and [^{35}S]GTPγS experiments performed in CHO_{hNOP} cell membranes, in calcium mobilization experiments performed in CHO_{hNOP} cells expressing the $G\alpha_{qi5}$ protein, and in N/OFQ sensitive isolated tissues. Finally, the *in vivo* actions of Compound 24 were assessed in mice using the tail withdrawal assay.

2. Materials and methods

2.1. Cell culture and membrane preparation

CHO_{hNOP} cells were cultured in Dulbecco Minimum Essential Medium (DMEM) and Ham F-12 (1:1) supplemented with 5% foetal calf serum, penicillin (100 IU/ml), Streptomycin (100 μg/ml) and Fungizone (2.5 μg/ml). Stock cultures were further supplemented with geneticin (G418, 200 μg/ml) and Hygromycin B (200 μg/ml) as described previously (McDonald et al., 2003). CHO_{hmu}, CHO_{hdelta}, CHO_{hkappa} and CHO_{hNOP} stably expressing the $G\alpha_{qi5}$ protein were generated as previously described (Camarda et al., 2009) and maintained in DMEM and Ham F-12 (1:1) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 200 μg/ml Geneticin, 100 μg/ml Hygromycin B. Cells were cultured at 37 °C in 5% carbon dioxide humidified air, and used when confluent. For binding experiments membranes were prepared from freshly harvested cell suspensions in Tris–HCl (50 mM), Mg^{2+} (5 mM) pH 7.4 ([^{3}H]N/OFQ binding experiments) or in Tris–HCl (50 mM), EGTA (0.2 mM) pH 7.4 ([^{35}S]GTPγS binding experiments) via homogenisation and centrifugation at 13,500 rpm for 10 min at 4 °C. The final protein concentration was determined according to Lowry et al. (1951).

2.2. Receptor binding experiments

2.2.1. [Leucyl-^{3}H]N/OFQ binding

5 μg of CHO_{hNOP} homogenate protein was incubated in 0.5 ml volumes of Tris–HCl (50 mM) buffer supplemented with 10 μM peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon), 0.5% bovine serum albumin (BSA), increasing concentrations of Compound 24 and approximately 200 pM [^{3}H]–N/OFQ. Total radiolabel bound was <<10%. Non-specific binding was determined in the presence of 1 μM unlabelled N/OFQ. In all experiments N/OFQ was included as a reference ligand. Reactions were incubated for 1 h at room temperature and terminated by vacuum filtration (Brandel Harvester) through Whatman GF/B filters soaked in 0.5% polyethylenimine. Radioactivity was determined after 8 h extraction in scintillation cocktail.

2.2.2. [^{3}H]-Diprenorphine binding

50 μg (CHO_{hmu}), 25 μg (CHO_{hdelta}) and 40 μg (CHO_{hkappa}) membrane protein were incubated in 0.5 ml buffer containing Tris–HCl (50 mM) pH 7.4, BSA (0.5%), ~0.7 nM [^{3}H]-Diprenorphine and increasing concentrations of naloxone and Compound 24. Non-specific binding was determined in the presence of 10 μM naloxone. Reactions were incubated at room temperature for 1 h. Harvesting and determination of radioactivity were as for [leucyl-^{3}H]N/OFQ binding.

2.3. [^{35}S]GTPγS binding experiments

20 μg of CHO_{hNOP} membranes were incubated in 0.5 ml buffer containing Tris–HCl (50 mM), EGTA (0.2 mM) $MgCl_2$ (1 mM), NaCl (100 mM), bacitracin (0.15 mM) peptidase inhibitors (as above), GDP (100 μM) and approximately 150 pM [^{35}S]GTPγS (McDonald et al., 2003). Compound 24 was pre-incubated for 15 min at 30 °C. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. The reaction was incubated for 1 h at 30 °C with gentle shaking and terminated by filtration through Whatman GF/B filters using a Brandel Harvester.

2.4. Calcium mobilization experiments

CHO_{hmu}, CHO_{hdelta}, CHO_{hkappa} and CHO_{hNOP} stably expressing the $G\alpha_{qi5}$ protein were seeded at a density of 40,000 cells/well into 96-well black, clear-bottom plates. After 24 h incubation the cells were loaded with medium supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. Afterwards the loading solution was aspirated and 100 μl/well of assay buffer: Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black (Aldrich) was added. Stock solutions (1 mM) of ligands were made in distilled water and stored at −20 °C. Serial dilutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA 94587, US), fluorescence changes were measured at room temperature. On-line additions were carried out in a volume of 50 μl/well.

2.5. Electrically stimulated isolated tissue experiments

Tissues were taken from male Swiss mice (30–35 g), albino guinea pigs (300–350 g) and Sprague-Dawley rats (300–350 g). The mouse and rat vas deferens and the guinea pig ileum were prepared as previously described (Bigoni et al., 1999; Calo et al., 1996). Tissues were suspended in 5 ml organ baths containing heated Krebs solution oxygenated with 95% O_2 and 5% CO_2. The bath temperature was set at 33 °C for mouse vas deferens and 37 °C for rat vas deferens and guinea pig ileum. Tissues were continuously stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. A resting tension of 0.3, 1 and 1.5 g was applied to the mouse and rat vas deferens, and guinea pig ileum, respectively. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006, UgoBasile s.r.l., Varese, Italy) and recorded with the PC based acquisition system Power Lab (ADInstrument, USA).

Following an equilibration period of 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration-response curves to N/OFQ were performed (0.5 log unit steps) in the absence or presence of Compound 24 (15 min pre-incubation time). For selectivity studies, in some experiments the delta selective agonist DPDPE was used in the mouse vas deferens while in others the mu selective agonist Dermorphin was used in the guinea pig ileum.

2.6. Tail withdrawal assay

Male Swiss albino mice weighing 25–30 g were used. Animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and Italian national regulations

Table 1
Affinities of Compound 24 and naloxone at NOP and classical opioid receptors expressed in CHO cell membranes.

Receptor	NOP	Mu	Delta	Kappa
Radioligand	[^{3}H]N/OFQ	[^{3}H]DPN	[^{3}H]DPN	[^{3}H]DPN
Naloxone[a]	<6	9.25 (9.04–9.46)	7.67 (7.59–7.75)	8.35 (8.20–8.50)
Compound 24	9.62 (9.47–9.77)	6.72 (6.43–6.97)	<6	6.47 (6.20–6.74)

[a] Naloxone affinities at classical opioid receptors are from Vergura et al. (2006). Data are mean ($CL_{95\%}$) of 3 separate experiments.

(D.L. 116/92). They were housed in 425×266×155-mm cages (Techniplast, Milan, Italy), fifteen animals/cage, under standard conditions (22 °C, 55% humidity, 12-h light/dark cycle, light on at 7:00 am) with food (MIL, standard diet; Morini, Reggio Emilia, Italy) and water *ad libitum* for at least 5 days before experiments began. Each mouse was used only once. I.c.v. (2 µl/mouse) or i.t (5 µl/mouse) injections were given according to the procedure described by Laursen and Belknap (1986) and Hylden and Wilcox (1980), respectively.

All experiments were started at 10:00 am and performed according to the procedure described previously in detail (Calo et al., 1998). Briefly, the mice were placed in a holder and the distal half of the tail was immersed in water at 48 °C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut-off time of 20 s was chosen to avoid tissue damage. For each experiment sixteen mice were used by randomly assigning four animals to each treatment group. The experiment was repeated four times; therefore, each experimental point shown in Figs. 5 and 6 is the mean of the results obtained in 16 mice. Tail-withdrawal latency was determined immediately before and 5, 15, 30, and 60 min after i.c.v. or i.t. injection of vehicle (saline) or N/OFQ (1 nmol) or endomorphin-1 (3 nmol, only i.t.). Compound 24 (10 mg/kg) or its vehicle (2% DMSO and 10% encapsin) were given i.p. 30 min before N/OFQ or endomorphin-1 administration. Increased and decreased tail withdrawal latencies compared with baseline indicated antinociceptive and pronociceptive effects, respectively.

2.7. Drugs

The peptides used in this study were prepared and purified as previously described (Guerrini et al., 1997). [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and endomorphin-1 were purchased from NeoMPS (Strasbourg, France). Compound 24 was synthesized in house following the procedures described in detail by Goto et al. (2006) with little modification. The structure and purity (>99%) of Compound 24 was confirmed by HPLC, mass spectrometry, proton and carbon NMR and polarimetry.

All tissues culture media and supplements were from Invitrogen (Paisley, UK). [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]DPN were from Perkin Elmer Life Sciences and [^{3}H]N/OFQ (75–133 Ci/mmol) from Amersham Biosciences. All other reagents were from Sigma Chemical Co. (Poole, U.K.) or E. Merck (Darmstadt, Germany) and were of the highest purity available.

For *in vitro* experiments Compound 24 was solubilized in dimethyl sulfoxide at a final concentration of 10 mM, and the successive dilutions were made in saline, whereas the other compounds were solubilized distilled water; stock solutions were kept at −20 °C until use. For *in vivo* studies, Compound 24 was dissolved in 2% DMSO and 10% encapsin (hydroxypropyl-beta-cyclodextrin) just before performing the experiment.

2.8. Data analysis and terminology

All data are expressed as means ± standard error of the mean (S.E.M.) of *n* experiments. For potency values 95% confidence limits were indicated. Data have been analyzed statistically using one-way ANOVA followed by Dunnett's test for multiple comparisons. Receptor binding data (Table 1) are expressed as pK_i derived from the Cheng and Prusoff (Cheng and Prusoff, 1973) equation:

$$pK_i = IC_{50}/(1 + ([R]/K_d))$$

where [*R*] is the concentration of the radiolabel and K_d is the radiolabel affinity for the receptor under investigation. The [^{3}H]N/OFQ K_d was 83 pM while those of [^{3}H]diprenorphine were 125, 323, and 134 pM at mu, delta, and kappa, respectively.

[^{35}S]GTPγS data are expressed as a stimulation factor i.e., the ratio between agonist-stimulated [^{35}S]GTPγS specific (minus NSB) binding and basal specific binding. Calcium mobilization data are expressed as fluorescence intensity units (FIU) in percent over the baseline. Isolated tissue data are expressed as percent of the twitch induced by electrical field stimulation.

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. Concentration response curve to agonists were fitted with the following equation:

$$\text{Effect} = \text{baseline} + (\text{Emax} - \text{baseline}) \div (1 + 10\text{^}((\text{LogEC}_{50} - X)\text{*HillSlope}))$$

where *X* is the agonist concentration.

Antagonist potencies have been calculated in different ways. In [^{35}S]GTPγS binding (Fig. 1) and mouse vas deferens (Fig. 3) pA_2 values were derived from the classical Schild protocol. For rat vas deferens

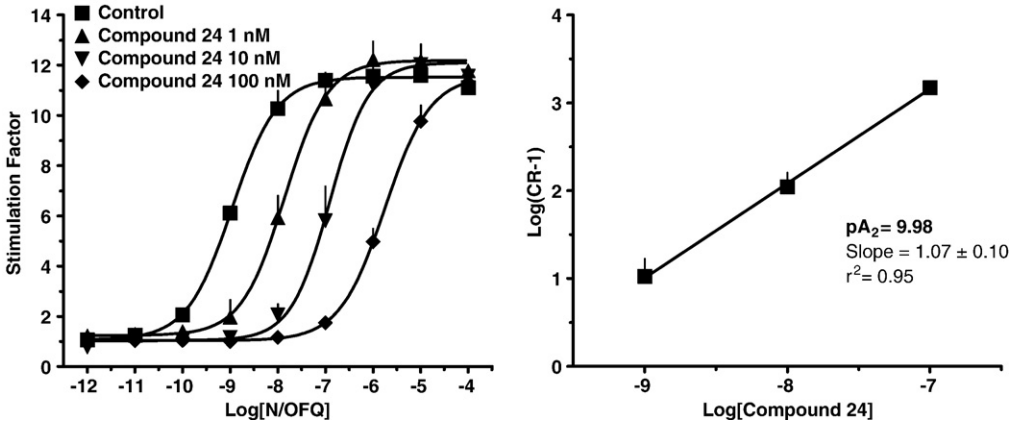

Fig. 1. Left panel: concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Compound 24 (1–100 nM) in the [^{35}S]GTPγS binding assay performed on CHO_{hNOP} cell membranes. The relative Schild Plot is shown in the right panel. Data are mean ± S.E.M. of 4 separate experiments.

and guinea pig ileum experiments (Fig. 4) pK_B values were derived from the Gaddum Schild equation:

$$K_B = ((CR - 1) / [antagonist])$$

assuming a slope value equal to unity, where CR indicate the ratio between agonist potency in the presence and absence of antagonist. For calcium mobilization experiments pK_B values were derived from inhibition response curves (Table 2) using the following equation:

$$K_B = IC_{50} / \left(\left[2 + ([A] / EC_{50})^n \right]^{1/n} - 1 \right)$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [*A*] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response and n is the Hill coefficient of the concentration response curve to the agonist (Kenakin, 2004). In addition the pK_B for Compound 24 evaluated at different concentrations against the concentration response curve to N/OFQ (Fig. 2) was calculated using the following equation:

$$K_B = [antagonist] / (slope - 1)$$

where slope is calculated from a double-reciprocal plot of equieffective concentrations of agonist in the absence and presence of antagonist (Kenakin, 2004).

For *in vivo* studies, raw data from tail withdrawal experiments were converted to the area under the time versus tail withdrawal latency curve (AUC min/s). AUC data for the time interval (0–15 and 0–30 min for i.c.v. and i.t. studies, respectively) was calculated and used for statistical analysis.

3. Results

3.1. Receptor binding

In receptor binding experiments performed on CHO_{hNOP} cell membranes Compound 24 displaced [^{3}H]N/OFQ in a concentration dependent manner showing subnanomolar affinity (pK_i 9.62, Table 1). Under the same experimental conditions, Compound 24 did not bind the delta receptor and showed low affinities for mu and kappa sites (pK_i 6.72 and 6.47, respectively). In contrast, the universal opioid receptor ligand naloxone did not bind the NOP receptor while showed the expected rack order of affinity at classical opioid receptors i.e., mu (pK_i 9.25)>kappa (pK_i 8.35)>delta (pK_i 7.67) (Table 1).

3.2. [^{35}S]GTPγS binding

In CHO_{hNOP} cell membranes N/OFQ stimulated [^{35}S]GTPγS binding in a concentration-dependent manner with a pEC_{50} value of 8.95 ± 0.05 and E_{max} of 11.71 ± 0.37 (Fig. 1, left panel). The antagonistic properties of Compound 24 were evaluated over the 1–100 nM concentration range, in order to obtain data for a Schild analysis. Compound 24 up to 10 μM did not elicit any stimulation of [^{35}S]GTPγS binding in CHO_{hNOP} cell membranes, however it produced a concentration dependent and parallel shift of the concentration response curve to N/OFQ without modifying the maximal effects induced by the agonist (Fig. 1, left panel). Schild analysis of the data (Fig. 1, right panel) demonstrated a linear ($r^2 = 0.95$) plot with a slope not significantly different from unity. The extrapolated pA_2 value was 9.98.

Table 2
Antagonist potencies of Compound 24 and naloxone evaluated in calcium mobilization experiments performed in CHO cells expressing NOP or classical opioid receptors and the $G\alpha_{qi5}$ protein.

Receptor	NOP	Mu	Delta	Kappa
Agonist	N/OFQ 10 nM	Dermorphin 100 nM	DPDPE 100 nM	Dynorphin A 100 nM
Naloxone	<6	9.09 (8.73–9.45)	7.32 (6.11–8.53)	7.14 (6.60–7.68)
Compound 24	9.03 (8.83–9.23)	<6	<6	<6

Data are mean ($CL_{95\%}$) of 4 separate experiments.

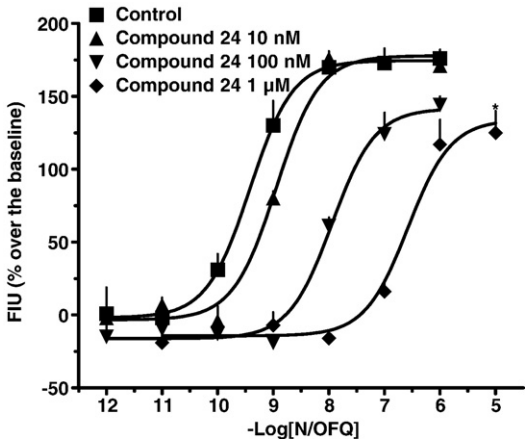

Fig. 2. Concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Compound 24 (10 nM–1 μM) in the calcium mobilization assay performed on CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. Data are mean ± S.E.M. of 4 separate experiments performed in duplicate. *$p<0.05$ versus control according to ANOVA followed by Dunnett's test.

3.3. Calcium mobilization

In CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein N/OFQ evoked a concentration dependent stimulation of calcium release (pEC_{50} 9.24 ($CL_{95\%}$ 9.10–9.38)). Compound 24 was inactive per se but in the range 0.01 nM–10 μM concentration-dependently inhibited calcium mobilization induced by 10 nM N/OFQ with a pK_B value of 9.03 ± 0.20 (Table 2). Naloxone was inactive up to 1 μM. To assess the selectively of action of Compound 24 similar experiments were performed in CHO cells stably expressing $G\alpha_{qi5}$ and classical opioid receptors. Dermorphin, DPDPE and Dynorphin A were used in these experiments as agonists for mu, delta and kappa receptors, respectively. They produced a concentration dependent stimulation of calcium with the following values of pEC_{50} and E_{max}:Dermorphin 7.93 ($CL_{95\%}$ 7.67–8.19), 196 ± 9%; DPDPE 8.82 ($CL_{95\%}$ 8.43–9.21), 130 ± 10%; Dynorphin A 8.47 ($CL_{95\%}$ 8.16–8.78) 174 ± 14%. Naloxone inhibited the effects of these agonists showing higher potency at mu (pK_B 9.09) than kappa (pK_B 7.14) and delta (pK_B 7.32) (Table 2). In contrast Compound 24 was inactive up to 1 μM against DPDPE, Dynorphin A, and Dermorphin (Table 2).

To investigate the type of antagonism produced by Compound 24 in calcium mobilization experiments a Schild analysis has been performed by testing this molecule at various concentrations (0.01, 0.1 and 1 μM) against the concentration-response curve to N/OFQ. As shown in Fig. 2, Compound 24 produced a rightward shift of the concentration-response curve to N/OFQ in a concentration dependent manner. However, the maximal effects elicited by N/OFQ appear to be slightly but significantly reduced by Compound 24. A pK_B value of 8.73 was derived from these experiments. It is worthy of mention that this value is close to that obtained in inhibition response experiments (9.03).

3.4. Electrically stimulated isolated tissue

Compound 24 was assessed against N/OFQ in the electrically stimulated mouse and rat vas deferens and guinea pig ileum. In the

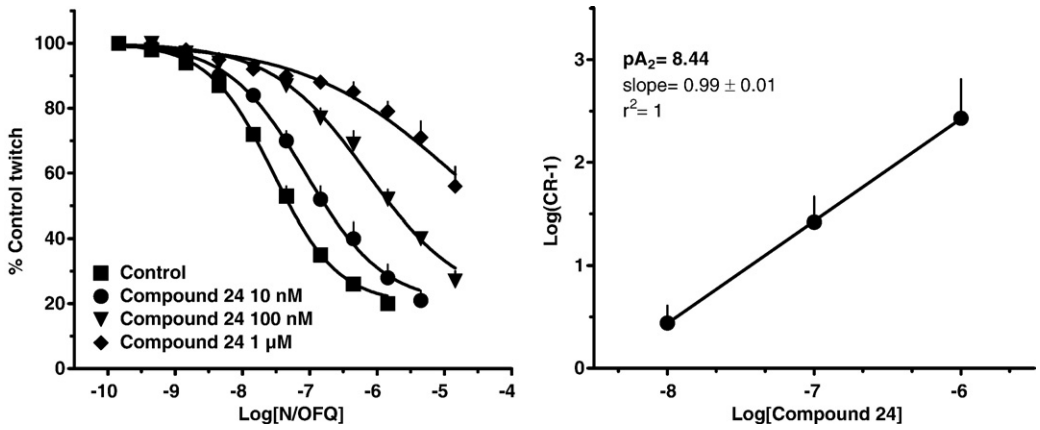

Fig. 3. Left panel: concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Compound 24 (10 nM–1 μM) in the electrically stimulated mouse vas deferens. The relative Schild Plot is shown in the right panel. Data are mean ± S.E.M. of 4 separate experiments.

mouse vas deferens N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner (pEC_{50} value 7.46, Fig. 3 left panel). Compound 24, tested over the concentration range 10 nM–1 μM, did not modify *per se* the electrically-induced twitches, but displaced to the right the concentration response curve to N/OFQ in a concentration dependent manner. Curves obtained in the presence of Compound 24 were parallel to the control (Fig. 3, left panel) with no modification of the agonist maximal effect (this parameter could not be estimated in the presence of Compound 24 1 μM because the concentration response curve to N/OFQ was still incomplete at 10 μM concentration of peptide). The corresponding Schild plot was linear ($r^2 = 1.00$) with a slope not significantly different from unity, yielding a pA_2 value of 8.44 (Fig. 3, right panel).

In the rat vas deferens and in guinea pig ileum Compound 24 was tested at the single concentration of 100 nM against the effects of N/OFQ. In both preparations the concentration response curves to N/OFQ obtained in the absence and presence of Compound 24 were parallel and reached similar maximal effects. The estimated pK_B values were 8.28 ± 0.12 and 9.12 ± 0.19 in the rat vas deferens (Fig. 4, left panel) and guinea pig ileum (Fig. 4, right panel), respectively. In these preparations, Compound 24 up to 1 μM was *per se* inactive.

Finally, Compound 24 at 1 μM did not modify the inhibitory effects of DPDPE in the mouse vas deferens (control: pEC_{50} (95% confidence limit) 8.38 (8.20–8.56), E_{max}, 98 ± 1%; 1 μM Compound 24: pEC_{50} 8.30 (7.80–8.80), E_{max}, 99 ± 1%) or those evoked by Dermorphin in the guinea pig ileum (control: pEC_{50} 8.52 (8.35–8.69), E_{max}, 98 ± 3%; 1 μM Compound 24: pEC_{50}, 8.51 (8.35–8.67), E_{max}, 95 ± 3%).

3.5. Tail withdrawal assay

In tail withdrawal experiments, mice injected with vehicle (either i.c.v. or i.t.) displayed tail withdrawal latencies of approximately 5 s that were stable over the time course of the experiment (Fig. 5). In line with previous studies, N/OFQ (1 nmol) applied i.c.v. significantly reduced tail withdrawal latency with a maximal effect (about 50% reduction in tail withdrawal latency) obtained at 5 min ($AUC_{[0-15\ min]}$ vehicle 77 ± 5; 1 nmol of N/OFQ 51 ± 3, $p<0.05$). The i.p. administration of Compound 24 up to 10 mg/kg did not modify, *per se*, tail withdrawal latencies ($AUC_{[0-15\ min]}$, 80 ± 7) but prevented the pronociceptive effects of the natural peptide ($AUC_{[0-15\ min]}$, 66 ± 5) (Fig. 5, left panel). On the other hand Compound 24 at 1 mg/kg did not modify the pronociceptive effect of i.c.v. N/OFQ (data not shown).

When the same dose of N/OFQ was administered i.t., a statistically significant antinociceptive effect was recorded ($AUC_{[0-30\ min]}$ vehicle, 165 ± 14; 1 nmol N/OFQ 346 ± 25, $p<0.05$) (Fig. 5, right panel). This antinociceptive effect was reduced by Compound 24 at 10 mg/kg ($AUC_{[0-30\ min]}$, 226 ± 25) (Fig. 5, right panel) but not at 1 mg/kg (data not shown).

In a separate set of experiments performed under the same experimental conditions, the selective mu receptor agonist endomorphin-1 produced after i.t. injection a clear antinociceptive effect ($AUC_{[0-30\ min]}$

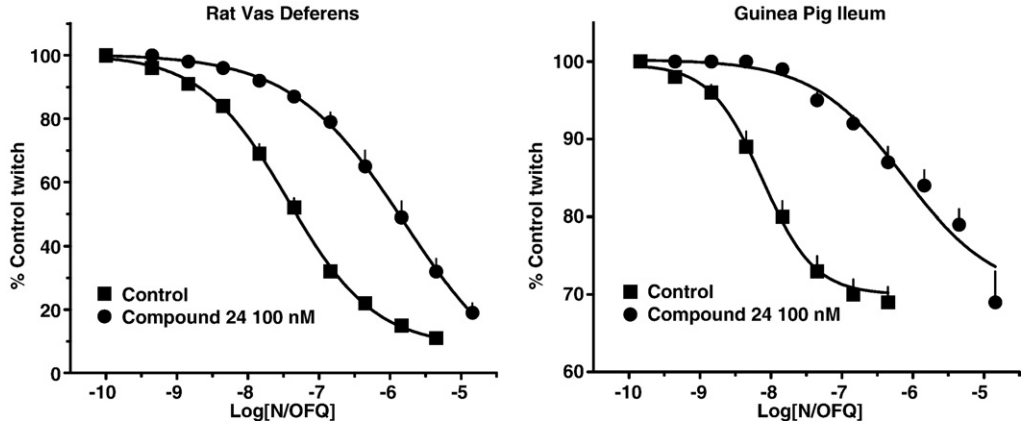

Fig. 4. Concentration response curves to N/OFQ obtained in the absence (control) and presence of 100 nM Compound 24 in the electrically stimulated rat vas deferens (left panel) and guinea pig ileum (right panel). Data are mean ± S.E.M. of 4 separate experiments.

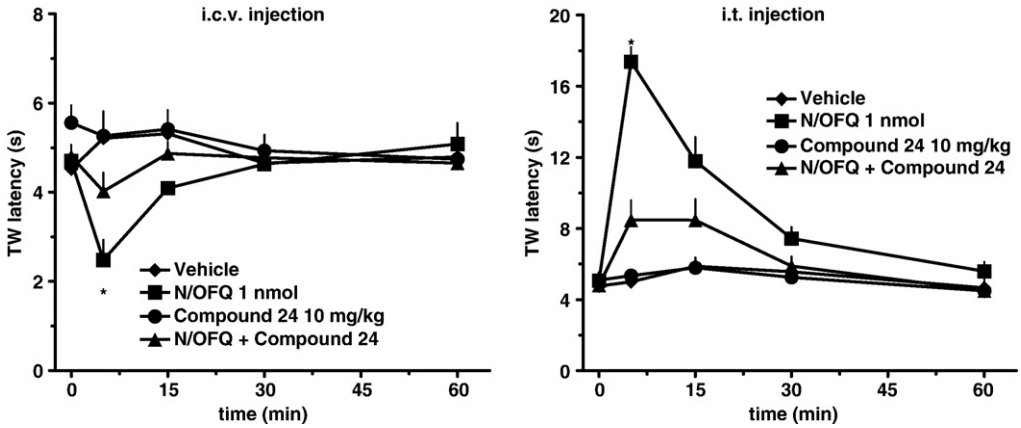

Fig. 5. Mouse tail withdrawal assay. Effects of Compound 24 (10 mg/kg i.p., 30 min pre-treatment) on the pronociceptive or antinociceptive effects induced by 1 nmol N/OFQ injected i.c.v. (left panel) or i.t. (right panel). Data are mean ± S.E.M. of 4 separate experiments.*$p<0.05$ versus vehicle according to ANOVA followed by the Dunnett's test.

vehicle, 152 ± 18; 3 nmol endomorphin-1 395 ± 31, $p<0.05$) (Fig. 6). Ten mg/kg of Compound 24 did not produce any effect per se ($AUC_{[0-30\ min]}$, 184 ± 17) and did not affect the antinociceptive effects elicited by endomorphin-1 ($AUC_{[0-30\ min]}$, 352 ± 35) (Fig. 6).

4. Discussion

The present study extend previous findings (Goto et al., 2006) demonstrating that Compound 24 binds with high affinity the NOP receptor and behaves as a pure and potent NOP receptor antagonist showing high selectivity over classical opioid receptor. These pharmacological features of Compound 24 were consistently observed in various assays and preparations expressing the human recombinant as well as the animal native receptors. In addition, the NOP selective antagonist properties of Compound 24 have been confirmed *in vivo* in mice subjected to the tail withdrawal assay. Therefore Compound 24 represents a valuable research tool that should be included in the class of selective NOP receptor antagonists and used in future target validations studies.

In receptor binding studies Compound 24 displayed very high affinity for the NOP receptor; in fact, the pK_i value calculated from the present experiments (9.62) is virtually superimposable to that previously reported by Goto et al. (2006) (pIC_{50} 9.57). These values of affinity are similar to that reported for SB-612111 and higher than that of J-113397 (Ozaki et al., 2000; Spagnolo et al., 2007; Zaratin et al., 2004). In functional studies performed on the human recombinant receptor ([^{35}S]GTPγS binding and calcium mobilization assays) and on animal native receptors from various species (mouse, rat, guinea pig) Compound 24 consistently behaved as a pure NOP receptor antagonist showing, in line with receptor binding studies, high values of potency (range 8.28–9.12). The only result which was out of this range is that obtained in [^{35}S]GTPγS binding studies where Compound 24 displayed a pA_2 of 9.98. However, this result, which is again superimposable to that obtained by Goto et al. (2006)(pIC_{50} 9.82), is expected on the basis of what we have observed with several NOP receptor antagonists, including the peptides [Nphe^1]N/OFQ(1–13)NH_2 and UFP-101 (Calo et al., 2002a, 2005), and the non peptides J-113397, Trap-101, and SB-612111 (Spagnolo et al., 2007; Trapella et al., 2006), that consistently showed, in this particular assay, values of potency approx 10 fold higher than in the other tests. As previously suggested (Spagnolo et al., 2007), this might be due to the higher receptor accessibility in membranes (where the [^{35}S]GTPγS binding assay is performed) than in whole cells or tissue preparations (where the other assays are performed). Despite this minor difference, very similar antagonist potency values were obtained in the different assays for Compound 24 demonstrating the recombinant and native as well as species-specific NOP receptors are similarly sensitive to this antagonist. This also applies to the NOP receptors expressed in rat periaqueductal gray slices (Yan-Yu and Chiou, 2008) and sympathetic neurons (Ruiz-Velasco et al., 2008) although no quantitative data were reported in these latter studies. The consistency of Compound 24 potency values among preparations and species corroborates previous findings obtained with peptide (Calo et al., 2000, 2002a,b, 2005) and non peptide (Bigoni et al., 2000; Ozaki et al., 2000; Spagnolo et al., 2007; Trapella et al., 2006; Zaratin et al., 2004) NOP antagonists and enable the following rank order of antagonist potency to be proposed: Compound 24 (≈8.5) = SB-612111 (≈8.5) > J-113397 (≈8.0) > UFP-101 (≈7.5) = Trap-101 (≈7.5) > [Nphe^1]N/OFQ(1–13)NH_2 (≈6.5) as NOP receptor fingerprint.

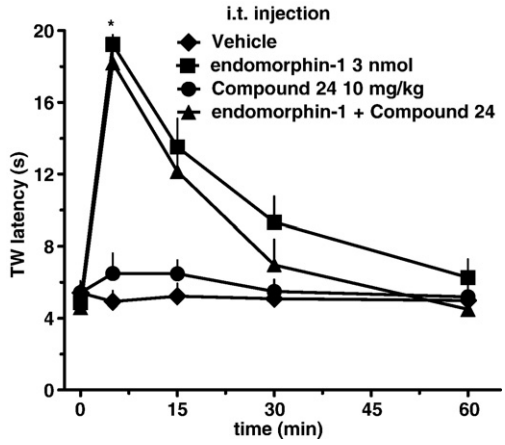

Fig. 6. Mouse tail withdrawal assay. Effects of Compound 24 (10 mg/kg i.p., 30 min pre-treatment) on the antinociceptive effects induced by 3 nmol emdomophin-1 injected i.t. Data are mean ± S.E.M. of 4 separate experiments. *$p<0.05$ versus vehicle according to ANOVA followed by the Dunnett's test.

With respect to the type of antagonism exerted by Compound 24, results obtained in the [^{35}S]GTPγS binding and mouse vas deferens assay by performing concentration response curve to N/OFQ in the presence of increasing concentrations of antagonist are clearly compatible with a competitive type of interaction between Compound 24 and N/OFQ. Similar results were obtained by testing Compound 24 in the calcium mobilization assay where a rightward displacement of the concentration response curve to N/OFQ was recorded in response to increasing concentration of antagonist. However in these experiments Compound 24 at the highest concentrations tested produced a slight but statistically significant reduction of N/OFQ maximal effect. This apparent insurmountable antagonism behaviour can be attributed to i) the transient nature of the calcium response that may not allow equilibrium between agonist–antagonist competition to be

reached thus generating depression of the agonist response in the presence of high concentrations of antagonist (Kenakin, 2004), ii) lack of stirring in the 96 well plate which is another source of hemiequilibrium conditions (Kenakin, 2004), iii) a combination of the two factors. The observation that a truly competitive antagonist produces a reduction of agonist maximal effects in calcium mobilization assay is not uncommon. For instance, the urotensin-II receptor antagonists urantide and UFP-803 competitively antagonized the contractile effects of urotensin-II in the rat aorta bioassay while depressed maximal responses to the agonist in calcium experiments performed on cells expressing the rat recombinant urotensin-II receptor (Camarda et al., 2006). On this basis, we propose to classify Compound 24 as a competitive NOP receptor antagonist.

Selectivity of action along with high affinity/potency and pure antagonist activity is another important feature of a valuable research tool. This property of Compound 24 has been evaluated in three sets of experiments over classical opioid receptors. In receptor binding studies Compound 24 displayed approximately 1000 fold selectivity over classical opioid receptors. A superimposable value of selectivity was found in functional studies performed measuring calcium mobilization in cells expressing the $G\alpha_{qi5}$ chimeric protein. Finally at least 300 fold selectivity of Compound 24 for NOP over mu and delta receptors was obtained in bioassay experiments. These data confirm the impressive selectivity profile of Compound 24 reported by Goto et al. (2006). Thus, in terms of selectivity over classical opioid receptor Compound 24 seems to be similar to SB-612111 (Spagnolo et al., 2007; Zaratin et al., 2004) and UFP-101 (Calo et al., 2002b) and certainly more selective then J-113397 (Ozaki et al., 2000; Spagnolo et al., 2007) for which NOP independent effects were reported *in vivo* (Koizumi et al., 2004). However more comprehensive selectivity studies are needed against a large panel of receptors and ion channels to firmly classifying Compound 24 as a highly selective NOP receptor antagonist.

Collectively, these *in vitro* data confirm and extend previous findings (Goto et al., 2006) demonstrating that Compound 24 is a pure, competitive, selective and potent NOP receptor antagonist. This excellent *in vitro* pharmacological profile is associated with good brain penetration after peripheral administration (Goto et al., 2006). Indeed, Compound 24 was reported to be active *in vivo* in mice where at 10 mg/kg it prevented the inhibitory effects on locomotor activity of a non peptide NOP agonist (Goto et al., 2006). Here we assessed the *in vivo* effects of Compound 24 in the mouse tail withdrawal assay. N/OFQ has been repeatedly demonstrated to exert in rodents pronociceptive and antinociceptive effects following supraspinal and spinal injection, respectively (Zeilhofer and Calo, 2003). The present results i.e., pronociceptive and antinociceptive response to 1 nmol N/OFQ given i.c.v. and i.t., are therefore in line with the literature. Both of these *in vivo* actions of N/OFQ are due to selective NOP receptor activation as consistently demonstrated by knockout (Nazzaro et al., 2007; Nishi et al., 1997) and receptor antagonist (UFP-101 (Calo et al., 2002b; Nazzaro et al., 2007), J-113397 (Ozaki et al., 2000; Ueda et al., 2000), SB-612111 (Rizzi et al., 2007; Zaratin et al., 2004)) studies. In line with these findings, Compound 24 at 10 mg/kg antagonised both the pronociceptive and antinociceptive effects evoked by N/OFQ when given spinally and supraspinally, respectively. Moreover, in line with *in vitro* findings, the antagonist action of Compound 24 *in vivo* is selective for the NOP receptor as demonstrated by the lack of effect of this molecule against the antinociceptive effects elicited by spinal injection of the mu selective agonist endomorphin-1. These results demonstrated, on the one hand, that Compound 24 is an effective and selective antagonist at NOP receptors regulating pain transmission *in vivo* and, on the other, that the effects of N/OFQ on pain transmission are exclusively due to NOP receptor activation. Interestingly, Compound 24 at 10 mg/kg counteracted rather than fully prevented the effects of 1 nmol N/OFQ. Under the same experimental conditions SB-612111 completely blocked the actions of the same dose of N/OFQ at ten fold lower doses (i.e., 1 mg/kg) (Rizzi et al., 2007). Therefore SB-612111 appeared to be more potent than Compound 24 *in vivo* in the mouse tail withdrawal assay. Since the *in vitro* potency of the two antagonists is similar (see above), it can be proposed that *in vivo* potency differences may derive from better pharmacokinetic properties of SB-612111 than Compound 24. However, this is merely speculation that required rigorous experimental validation. Finally, the systemic administration of Compound 24 at pharmacologically active doses did not modify per se tail withdrawal latencies. Again this is in line with previous findings obtained with both receptor antagonists (Ozaki et al., 2000; Rizzi et al., 2007; Ueda et al., 2000; Zaratin et al., 2004) and mice knockout for the NOP receptor gene (Nazzaro et al., 2007; Nishi et al., 1997), and indicates that the endogenous N/OFQ-NOP receptor system is not activated by the mild and acute stimulus employed for evoking the nociceptive response in this assay. However the endogenous N/OFQergic signalling can be activated using more intense and prolonged nociceptive stimuli such as formalin. In fact in the mouse formalin assay the spinal antinociceptive action of endogenous N/OFQ seems to prevail on the supraspinal pronociceptive effect as indicated by the pronociceptive phenotype of NOP knockout mice (Depner et al., 2003; Rizzi et al., 2006) (recently confirmed in the acetic acid-induced writhing test by Rizzi et al. (2008)) and by the pronociceptive effects elicited by NOP antagonists e.g. systemic J-113397 (Rizzi et al., 2006).

In conclusion the present study demonstrated that Compound 24 is a pure, competitive, selective and potent NOP receptor antagonist. The NOP antagonist properties of Compound 24 were demonstrated in a large panel of *in vitro* assays and *in vivo* in the mouse tail withdrawal assay. Based on its pharmacological profile Compound 24 should be included (together with J-113397, SB-612111 and UFP-101) in the list of potent and selective NOP receptor antagonists to be tested in future target validation studies for firmly defining their therapeutic potential as innovative drugs for treating depression, Parkinson's disease and possibly sepsis.

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References

Bigoni, R., Giuliani, S., Calo, G., Rizzi, A., Guerrini, R., Salvadori, S., Regoli, D., Maggi, C.A., 1999. Characterization of nociceptin receptors in the periphery: in vitro and in vivo studies. Naunyn-Schmiedebergs Arch. Pharmacol. 359, 160–167.

Bigoni, R., Calo, G., Rizzi, A., Guerrini, R., De Risi, C., Hashimoto, Y., Hashiba, E., Lambert, D.G., Regoli, D., 2000. In vitro characterization of J-113397, a non-peptide nociceptin/orphanin FQ receptor antagonist. Naunyn-Schmiedebergs Arch. Pharmacol. 361, 565–568.

Calo, G., Guerrini, R., Bigoni, R., Rizzi, A., Marzola, G., Okawa, H., Bianchi, C., Lambert, D.G., Salvadori, S., Regoli, D., 2000. Characterization of [Nphe(1)]nociceptin(1-13)NH(2), a new selective nociceptin receptor antagonist. Br. J. Pharmacol. 129, 1183–1193.

Calo, G., Rizzi, A., Bogoni, G., Neugebauer, V., Salvadori, S., Guerrini, R., Bianchi, C., Regoli, D., 1996. The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. Eur. J. Pharmacol. 311, R3–5.

Calo, G., Rizzi, A., Marzola, G., Guerrini, R., Salvadori, S., Beani, L., Regoli, D., Bianchi, C., 1998. Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay. Br. J. Pharmacol. 125, 373–378.

Calo, G., Rizzi, A., Bigoni, R., Guerrini, R., Salvadori, S., Regoli, D., 2002a. Pharmacological profile of nociceptin/orphanin FQ receptors. Clin. Exp. Pharmacol. Physiol. 29, 223–228.

Calo, G., Rizzi, A., Rizzi, D., Bigoni, R., Guerrini, R., Marzola, G., Marti, M., McDonald, J., Morari, M., Lambert, D.G., Salvadori, S., Regoli, D., 2002b. [Nphe1,Arg14,Lys15] nociceptin-NH2, a novel potent and selective antagonist of the nociceptin/orphanin FQ receptor. Br. J. Pharmacol. 136, 303–311.

Calo, G., Guerrini, R., Rizzi, A., Salvadori, S., Burmeister, M., Kapusta, D.R., Lambert, D.G., Regoli, D., 2005. UFP-101, a peptide antagonist selective for the nociceptin/orphanin FQ receptor. CNS Drug Rev. 11, 97–112.

Camarda, V., Spagnol, M., Song, W., Vergura, R., Roth, A.L., Thompson, J.P., Rowbotham, D.J., Guerrini, R., Marzola, E., Salvadori, S., Cavanni, P., Regoli, D., Douglas, S.A., Lambert, D.G., Calo, G., 2006. In vitro and in vivo pharmacological characterization of the novel UT receptor ligand [Pen(5),DTrp(7),Dab(8)]urotensin II(4-11) (UFP-803). Br. J. Pharmacol. 147, 92–100.

Camarda, V., Fischetti, C., Molinari, P., Ambrosio, C., Kostenis, E., Regoli, D., Guerrini, R., Salvadori, S., Calo, G., 2009. Pharmacological characterization of nociceptin/orphanin FQ receptors coupled with calcium signaling via the chimeric protein Galphaqi5. Naunyn Schmiedebergs Arch Pharmacol. 379, 599–607.

Carvalho, D., Petronilho, F., Vuolo, F., Machado, R.A., Constantino, L., Guerrini, R., Calo, G., Gavioli, E.C., Streck, E.L., Dal-Pizzol, F., 2008. The nociceptin/orphanin FQ-NOP receptor antagonist effects on an animal model of sepsis. Intensive Care Med. 34, 2284–2290.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Chiou, L.C., Liao, Y.Y., Fan, P.C., Kuo, P.H., Wang, C.H., Riemer, C., Prinssen, E.P., 2007. Nociceptin/orphanin FQ peptide receptors: pharmacology and clinical implications. Curr. Drug Targets 8, 117–135.

Depner, U.B., Reinscheid, R.K., Takeshima, H., Brune, K., Zeilhofer, H.U., 2003. Normal sensitivity to acute pain, but increased inflammatory hyperalgesia in mice lacking the nociceptin precursor polypeptide or the nociceptin receptor. Eur. J. Neurosci. 17, 2381–2387.

Gavioli, E.C., Calo, G., 2006. Antidepressant- and anxiolytic-like effects of nociceptin/orphanin FQ receptor ligands. Naunyn-Schmiedebergs Arch. Pharmacol. 372, 319–330.

Gavioli, E.C., Marzola, G., Guerrini, R., Bertorelli, R., Zucchini, S., De Lima, T.C., Rae, G.A., Salvadori, S., Regoli, D., Calo, G., 2003. Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: pharmacological and genetic evidences from the mouse forced swimming test. Eur. J. Neurosci. 17, 1987–1990.

Gavioli, E.C., Vaughan, C.W., Marzola, G., Guerrini, R., Mitchell, V.A., Zucchini, S., De Lima, T.C., Rae, G.A., Salvadori, S., Regoli, D., Calo, G., 2004. Antidepressant-like effects of the nociceptin/orphanin FQ receptor antagonist UFP-101: new evidence from rats and mice. Naunyn-Schmiedebergs Arch. Pharmacol. 369, 547–553.

Goto, Y., Arai-Otsuki, S., Tachibana, Y., Ichikawa, D., Ozaki, S., Takahashi, H., Iwasawa, Y., Okamoto, O., Okuda, S., Ohta, H., Sagara, T., 2006. Identification of a novel spiropiperidine opioid receptor-like 1 antagonist class by a focused library approach featuring 3D-pharmacophore similarity. J. Med. Chem. 49, 847–849.

Guerrini, R., Calo, G., Rizzi, A., Bianchi, C., Lazarus, L.H., Salvadori, S., Temussi, P.A., Regoli, D., 1997. Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1-13)-peptide amide. J. Med. Chem. 40, 1789–1793.

Hylden, J.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 17, 313–316.

Kenakin, T., 2004. A Pharmacology Primer. Elsevier Academic Press, San Diego.

Koizumi, M., Sakoori, K., Midorikawa, N., Murphy, N.P., 2004. The NOP (ORL1) receptor antagonist Compound B stimulates mesolimbic dopamine release and is rewarding in mice by a non-NOP-receptor-mediated mechanism. Br. J. Pharmacol. 143, 53–62.

Lambert, D.G., 2008. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. Nat. Rev. Drug Discov. 7, 694–710.

Laursen, S.E., Belknap, J.K., 1986. Intracerebroventricular injections in mice. Some. methodological refinements. J. Pharmacol. Methods 16, 355–357.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marti, M., Mela, F., Guerrini, R., Calo, G., Bianchi, C., Morari, M., 2004a. Blockade of nociceptin/orphanin FQ transmission in rat substantia nigra reverses haloperidol-induced akinesia and normalizes nigral glutamate release. J. Neurochem. 91, 1501–1504.

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., 2004b. Blockade of nociceptin/orphanin FQ receptor signaling in rat substantia nigra pars reticulata stimulates nigrostriatal dopaminergic transmission and motor behavior. J. Neurosci. 24, 6659–6666.

Marti, M., Mela, F., Fantin, M., Zucchini, S., Brown, J.M., Witta, J., Di Benedetto, M., Buzas, B., Reinscheid, R.K., Salvadori, S., Guerrini, R., Romualdi, P., Candeletti, S., Simonato, M., Cox, B.M., Morari, M., 2005. Blockade of nociceptin/orphanin FQ transmission attenuates symptoms and neurodegeneration associated with Parkinson's disease. J. Neurosci. 25, 9591–9601.

Marti, M., Trapella, C., Viaro, R., Morari, M., 2007. The nociceptin/orphanin FQ receptor antagonist J-113397 and L-DOPA additively attenuate experimental parkinsonism through overinhibition of the nigrothalamic pathway. J. Neurosci. 27, 1297–1307.

McDonald, J., Calo, G., Guerrini, R., Lambert, D.G., 2003. UFP-101, a high affinity antagonist for the nociceptin/orphanin FQ receptor: radioligand and GTPgamma (35)S binding studies. Naunyn-Schmiedebergs Arch. Pharmacol. 367, 183–187.

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., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377, 532–535.

Nazzaro, C., Rizzi, A., Salvadori, S., Guerrini, R., Regoli, D., Zeilhofer, H.U., Calo, G., 2007. UFP-101 antagonizes the spinal antinociceptive effects of nociceptin/orphanin FQ: behavioral and electrophysiological studies in mice. Peptides 28, 663–669.

Nishi, M., Houtani, T., Noda, Y., Mamiya, T., Sato, K., Doi, T., Kuno, J., Takeshima, H., Nukada, T., Nabeshima, T., Yamashita, T., Noda, T., Sugimoto, T., 1997. Unrestrained nociceptive response and disregulation of hearing ability in mice lacking the nociceptin/orphaninFQ receptor. EMBO J. 16, 1858–1864.

Ozaki, S., Kawamoto, H., Itoh, Y., Miyaji, M., Azuma, T., Ichikawa, D., Nambu, H., Iguchi, T., Iwasawa, Y., Ohta, H., 2000. In vitro and in vivo pharmacological characterization of J-113397, a potent and selective non-peptidyl ORL1 receptor antagonist. Eur. J. Pharmacol. 402, 45–53.

Redrobe, J.P., Calo, G., Regoli, D., Quirion, R., 2002. Nociceptin receptor antagonists display antidepressant-like properties in the mouse forced swimming test. Naunyn-Schmiedebergs Arch. Pharmacol. 365, 164–167.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma Jr., F.J., Civelli, O., 1995. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270, 792–794.

Rizzi, A., Nazzaro, C., Marzola, G., Zucchini, S., Trapella, C., Guerrini, R., Zeilhofer, H.U., Regoli, D., Calo, G., 2006. Endogenous nociceptin/orphanin FQ signalling produces opposite spinal antinociceptive and supraspinal pronociceptive effects in the mouse formalin test: pharmacological and genetic evidences. Pain 124, 100–108.

Rizzi, A., Gavioli, E.C., Marzola, G., Spagnolo, B., Zucchini, S., Ciccocioppo, R., Trapella, C., Regoli, D., Calo, G., 2007. Pharmacological characterization of the nociceptin/orphanin FQ receptor antagonist SB–612111 [(-)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocycloheptен-5-ol]: in vivo studies. J. Pharmacol. Exp. Ther. 321, 968–974.

Rizzi, A., Marzola, G., Guerrini, R., Salvadori, S., Regoli, D., Calo, G., 2008. Antinociceptive effects of nociceptin/orphanin FQ receptor agonists in the mouse writhing test in: Society of Neuroscience Abstract 269.215 Washington, DC, USA. November, 15–19.

Ruiz-Velasco, V., Trapella, C., Calo, G., Margas, W., 2008. Pharmacology of constitutively active NOP opioid receptors heterologously expressed in rat sympathetic neurons, in: Society for Neuroscience, Abstract 533.9. Washington, DC, USA. November, 15–19.

Spagnolo, B., Carra, G., Fantin, M., Fischetti, C., Hebbes, C., McDonald, J., Barnes, T.A., Rizzi, A., Trapella, C., Fanton, G., Morari, M., Lambert, D.G., Regoli, D., Calo, G., 2007. Pharmacological characterization of the nociceptin/orphanin FQ receptor antagonist SB-612111 [(−)-cis-1-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol]: in vitro studies. J. Pharmacol. Exp. Ther. 321, 961–967.

Trapella, C., Guerrini, R., Piccagli, L., Calo, G., Carra, G., Spagnolo, B., Rubini, S., Fanton, G., Hebbes, C., McDonald, J., Lambert, D.G., Regoli, D., Salvadori, S., 2006. Identification of an achiral analogue of J-113397 as potent nociceptin/orphanin FQ receptor antagonist. Bioorg. Med. Chem. 14, 692–704.

Ueda, H., Inoue, M., Takeshima, H., Iwasawa, Y., 2000. Enhanced spinal nociceptin receptor expression develops morphine tolerance and dependence. J. Neurosci. 20, 7640–7647.

Vergura, R., Valenti, E., Hebbes, C.P., Gavioli, E.C., Spagnolo, B., McDonald, J., Lambert, D.G., Balboni, G., Salvadori, S., Regoli, D., Calo, G., 2006. Dmt-Tic-NH-CH2-Bid (UFP-502), a potent DOP receptor agonist: in vitro and in vivo studies. Peptides 27, 3322–3330.

Viaro, R., Sanchez-Pernaute, R., Marti, M., Trapella, C., Isacson, O., Morari, M., 2008. Nociceptin/orphanin FQ receptor blockade attenuates MPTP-induced Parkinsonism. Neurobiol. Dis. 30, 430–438.

Visanji, N.P., de Bie, R.M., Johnston, T.H., McCreary, A.C., Brotchie, J.M., Fox, S.H., 2008. The nociceptin/orphanin FQ (NOP) receptor antagonist J-113397 enhances the effects of levodopa in the MPTP-lesioned nonhuman primate model of Parkinson's disease. Mov Disord. 23, 1922–1925.

Williams, J.P., Thompson, J.P., Young, S.P., Gold, S.J., McDonald, J., Rowbotham, D.J., Lambert, D.G., 2008. Nociceptin and urotensin-II concentrations in critically ill patients with sepsis. Br. J. Anaesth. 100, 810–814.

Yan-Yu, L., Chiou, L.C., 2008. Effect of compound 24, a novel nociceptin/orphanin FQ (NOP) receptor antagonist, on NOP receptor-mediated k+ channel activation in rat periaqueductal gray slices. Society for Neuroscience, Abstract 826.14 Washington, DC, USA. November, 15–19.

Zaratin, P.F., Petrone, G., Sbacchi, M., Garnier, M., Fossati, C., Petrillo, P., Ronzoni, S., Giardina, G.A., Scheideler, M.A., 2004. Modification of nociception and morphine tolerance by the selective opiate receptor-like orphan receptor antagonist (−)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9- tetrahydro-5H-benzocyclohepten-5-ol (SB-612111). J. Pharmacol. Exp. Ther. 308, 454–461.

Zeilhofer, H.U., Calo, G., 2003. Nociceptin/orphanin FQ and its receptor-potential targets for pain therapy? J. Pharmacol. Exp. Ther. 306, 423–429.