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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