Modification of Nociception and Morphine Tolerance by the Selective Opiate Receptor-Like Orphan Receptor Antagonist \((-\text{cis})\)-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl][methyl]-6,7,8,9-tetrahydro-5\(H\)-benzocyclohepten-5-ol (SB-612111)

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ABSTRACT

\((-\text{cis})\)-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl][methyl]-6,7,8,9-tetrahydro-5\(H\)-benzocyclohepten-5-ol (SB-612111) is a novel human opiate receptor-like orphan receptor (ORL-1) antagonist that has high affinity for the clonal human ORL-1 receptor (hORL-1) with a high degree of structural homology to the classical opioid receptors (Mollereau et al., 1994). Despite the high similarity in sequence between nociceptin and other opioid peptides, notably dynorphin A, nociceptin does not interact with the ORL-1 receptor. Nociceptin does not interact with \(\mu\), \(\delta\), or \(\kappa\) opioid receptors, and opioid peptides do not interact with the ORL-1 receptor (Meunier et al., 1995). Activation of the ORL-1 receptor with nociceptin can enhance cellular \(K^+\) conductance (Vaughan et al., 1997), inhibit \(Ca^{2+}\) currents associated with \(N\), \(L\), and \(P/Q\) calcium channel activation (Knoflach et al., 1996; Connor and Christie, 1998), and block cellular cAMP production (Butour et al., 1997). These signaling mechanisms are represented in areas of the nervous system that are crucial for pain transmission, and nociceptin can act via these pathways to influence, for example, glutamate and GABA release in the periaqueductal gray (Vaughan et al., 1997) and neuronal activity in the dorsal root of the spinal cord (Lai et al., 1997). Furthermore, peptide antagonists of the ORL-1 receptor such as \([N\text{-Phe}^1\text{nociceptin}(1-13)\text{NH}_2\text{]}\) and UFP-101 have been shown to inhibit presynaptic \(5\text{-hydroxytryptamine}\)

Nociceptin (orphanin FQ) is a 17-amino acid peptide identified as a potent endogenous agonist for the opiate receptor-like orphan receptor (ORL-1), a G protein-coupled receptor with a high degree of structural homology to the classical opioid receptors (Mollereau et al., 1994). Despite the high similarity in sequence between nociceptin and other opioid peptides, notably dynorphin A, nociceptin does not interact with the ORL-1 receptor. Nociceptin does not interact with \(\mu\), \(\delta\), or \(\kappa\) opioid receptors, and opioid peptides do not interact with the ORL-1 receptor (Meunier et al., 1995). Activation of the ORL-1 receptor with nociceptin can enhance cellular \(K^+\) conductance (Vaughan et al., 1997), inhibit \(Ca^{2+}\) currents associated with \(N\), \(L\), and \(P/Q\) calcium channel activation (Knoflach et al., 1996; Connor and Christie, 1998), and block cellular cAMP production (Butour et al., 1997). These signaling mechanisms are represented in areas of the nervous system that are crucial for pain transmission, and nociceptin can act via these pathways to influence, for example, glutamate and GABA release in the periaqueductal gray (Vaughan et al., 1997) and neuronal activity in the dorsal root of the spinal cord (Lai et al., 1997). Furthermore, peptide antagonists of the ORL-1 receptor such as \([N\text{-Phe}^1\text{nociceptin}(1-13)\text{NH}_2\text{]}\) and UFP-101 have been shown to inhibit presynaptic \(5\text{-hydroxytryptamine}\)
mine and noradrenaline release in the neocortex (Marti et al., 2003).

Although nociceptin initiates signaling via the ORL-1 receptor in a manner that is similar to activation of the classical μ-, δ-, and κ-opioid receptors, its pharmacological effects can differ significantly. Nociceptin can have an antioioid action and when administered into the brain of mice potently antagonizes analgesia induced by systemic and intracerebroventricular (i.c.v.) morphine (Mogil et al., 1996). At the spinal level, intrathecal injection of low concentrations of nociceptin in mice causes allodynia and hyperalgesia (Hara et al., 1997), whereas at higher concentrations this peptide can cause analgesia (King et al., 1997; Tian et al., 1997). These effects are mediated at both a presynaptic site of action in different nerve terminals, and at a postsynaptic site of action on spinal interneurons (Neal et al., 1999). Ko et al. (2002) have further demonstrated a peripheral nervous system role of ORL-1 receptors in regulating thermal antinociception in primates.

These different activities of nociceptin suggest the existence of multiple activation pathways, making it difficult to predict the overall effects of systemically available ligands that can activate or antagonize the ORL-1 receptor. ORL-1 receptor knockout mice display a higher level of sensitivity to nociceptin treatment (Mitchell et al., 2000), whereas ORL-1 receptor knockout mice maintain a higher level of sensitivity to morphine. Importantly, we further demonstrate that SB-612111 can reactivate mice to morphine in animals that had been chronically treated with opiate, suggesting utility of this class of ORL-1 receptor antagonist in prolonging the analgesic action of morphine.

**Materials and Methods**

**Materials**

The following drugs and chemicals were obtained by the source indicated: media and sera (Invitrogen, Carlsbad, CA); [3H][d-Ala²,N-Me-Phe⁶,Gly⁵-ol]-enkephalin ([3H]DAMGO, 60 Ci/mmol) and [3H][d-Ala²,d-Leu⁵]-enkephalin (55 Ci/mmol) (New England Nuclear, Brussels, Belgium); [3H](U-69593 (60 Ci/mmol) and [3H]nociceptin (156 Ci/mmol) (Amersham, Italia Srl, Milan, Italy); naloxone hydrochloride and morphine hydrochloride (S.A.L.A.R.S., Como, Italy); [d-Pen²,d-Pen⁵]-enkephalin (DPDPE) and DAMGO (Sigma, Milan, Italy); BRL 52656 and U-69593 were purchased from Sigma/RBI (Natick, MA); and nociception was from Saxon Biochemical GmbH (Hannover, Germany). All other compounds used have been synthesized in-house.

**In Vitro Pharmacological Assays**

**Cell Culture.** Clonal cell lines stably expressing the human ORL-1, μ-opioid (hMOR), δ-opioid (hDOR), and κ-opioid receptor were established to allow the assessment of ligand binding affinities in radioreceptor assays specific for each receptor. Cell cultures were routinely maintained at 37°C in a humidified atmosphere containing 5% CO₂. Chinese hamster ovary (CHO) cells [317, obtained from ETCC (European Collection of Cell Cultures, Salisbury, UK)] were grown in suspension in 10179-2 culture medium (GlaxoSmithKline, Harlow, UK) containing 10% (v/v) fetal bovine serum (FBS) and 0.05% (v/v) pluronic acid (F68). Human embryonic kidney (HEK) 293 cells (5210602, obtained from ETCC) were grown in monolayer in Eagle’s minimal essential medium supplemented with 10% (v/v) FBS and 2 mM L-glutamine. hORL-1, hDOR, and hMOR receptor cDNAs were expressed in CHO cells and hKOR receptor cDNA in HEK293 cells, after integration into a pCDN expression vector (GlaxoSmithKline). Subclones stably expressing receptor were selected by growth in the absence of nucleosides (CHO) or by resistance to G-418 (HEK293). Subclones expressing high levels of radioligand binding were selected for further characterization.

**Membrane Preparation.** Membranes were prepared for use in radioligand binding assays by lysis in hypotonic phosphate buffer (approximately 30 × 10⁶ cells/30-ml tube), collected by centrifugation (1200 rpm, ca. 800g, 5 min), resuspended in 10 mM potassium phosphate buffer, pH 7.2 (buffer A), and centrifuged at 40,000g, 10 min. The pellets obtained were resuspended in the same volume of buffer A, incubated on ice for 20 min, and centrifuged at 1200 rpm, 5 min, saving the supernatants. The low-speed pellets were resuspended in buffer A again and the last step was repeated two more times, saving the supernatants each time. The low-speed supernatants were pooled and centrifuged at high speed at 4°C. The pellets obtained were resuspended in buffer A containing 0.32 M sucrose and 5 mM EDTA. The membranes were stored in this buffer until use at −80°C at a concentration of 2 to 5 mg protein/ml (ca. 10 × 10⁶ cells/ml).

**Measurement of Specific Radioligand Binding.** The radioligands [3H]DAMGO, [3H][d-Ala²,d-Leu⁵]-enkephalin, [3H]U-69593, and [3H]nociceptin were used to label μ, δ, κ, and ORL-1 receptors, respectively (Gillian and Kosterlitz, 1982; Petritello et al., 1989; Wang et al., 1994; Dooley et al., 1997). μ- and δ-Opioid receptor binding studies were performed in 25 mM potassium phosphate buffer, pH 7.4, in 96-well polystyrene square plates in a final volume of 0.7 ml. μ-Opioid and ORL-1 receptor binding studies were performed in 25 mM potassium phosphate buffer, pH 7.4, containing 3 mM MgCl₂.
in a final volume of 0.5 ml. The protease inhibitors bacitracin (100 
µg/ml), leupeptin (4 µg/ml), and chymostatin (2 µg/ml) were added to 
the incubation buffer of the ORL-1 receptor assay to prevent radioli-
gand degradation. Nonspecific binding was determined in the pres-
ence of 10 nM naloxone (-µ, -δ, and -κ-opioid receptor assays) or 10 
µM nociceptin (ORL-1 receptor assay). Incubation was carried out 
for 60 min at 25°C. The reaction was terminated by filtration using 
a Packard Filtermate harvester with GF/B Unifilter plates pre-
treated with buffer (-µ and -δ-opioid receptor assays) or 0.3% poly-
ethyleneimine (-κ-opioid and ORL-1 receptor assays). After filtration, 
Unifilter plates were dried, each well filled with 50 µl of Packard 
Microscint 30, and radioactivity was counted by a Packard TopCount 
NXT. Competition experiments were performed using a membrane 
concentration of 5 to 20 µg protein/ml and radioligand concentra-
tions close to the experimental Kd of each radioligand, as obtained 
from saturation binding studies. IC50 values were determined as 
described by Leatherbarrow (1990) using the nonlinear least-squares 
fitting program GraFit (Erlilicus Software Limited, Horley, UK) 
and results transformed into Kd values using the Cheng-Prusoff 
equation (Cheng and Prusoff, 1973).

In Vitro Assay of Receptor and Enzyme Interactions. 

SB-612111 was tested at a concentration of 10 µM by CEREP (Le 
Bois L’Èveque, France) in a broad profile of radioligand binding 
assays specific for different classes of receptors, ion channels, and 
enzymes. SB-612111 was initially dissolved in dimethyl sulfoxide to 
yield a concentration of 10 mM. Further dilutions of the compound 
for testing were in water. Assays were performed in duplicate and 
results expressed as the percentage of inhibition of specific radioli-
gand binding or specific enzyme activity. Affinity values (Ki) were 
measured using the Cheng-Prusoff equation for each receptor assay for 
which a significant interaction was detected.

Measurement of Receptor-Mediated Cellular Signaling. Re-
ceptor signaling studies were performed using a cAMP responsive 
CRE-luciferase-gene reporter assay, as described previously by Gar-
nier et al. (2003). Receptor expressing cell lines were generated by 
stably expressing plasmid containing cDNA for the human ORL-1 
receptor (pcDNA 3.1/Hygro/ORL1) or µ-opioid receptor (pcDNA 3.1/ 
Hygro/MOR) in a HEK293-Luc cell line derived from HEK293 cells 
and stably expressing a cAMP-responsive luciferase gene reporter 
construct. Recombinant clones expressing the hORL-1 receptor (HL-
ORL-1) or human µ-opioid receptor (HL-MOR) were obtained by 
selection using zeocin (400 µg/ml) and hygromycin (300 µg/ml), re-
spectively. HL-ORL-1 receptor and HL-MOR clones that expressed 
high levels of mRNA for each receptor were chosen for use in the 
signaling assays. These cell lines were grown in Eagle’s minimal 
esential medium culture medium containing 2 mM l-glutamine, 1% 
(w/v) non-essential amino acids, 0.4 mg/ml active genicin, and 0.4 
g/ml zeocin (HL-ORL) or 0.3 mg/ml hygromycin (HL-MOR), sup-
plemented with 10% (v/v) FBS (Invitrogen). Cells were incubated at 
37°C (5% CO2) on Falcon plastic culture dishes precoated with poly-
d-lysine (Sigma).

The luciferase-gene reporter assay was performed in 96-well for-
matic. Briefly, cells were plated in white Packard CulturPlates (Mil-
lipore S.p.A., Milan, Italy) at a density of 105 cells/well in phenol 
red-free medium. Twenty-four hours after seeding, cells were preinc-
cubated for 30 min in the presence of 0.5 mM phosphodiesterase 
inhibitor 3-isobutyl-1-methylxantine, as a means of inhibiting cAMP 
breakdown by endogenous phosphodiesterase activity. Cells were 
further incubated for 4 h in the presence of forskolin and test drugs 
at various concentrations, in a final volume of 100 µl. All compounds 
were freshly dissolved as 1 mM solutions in ethanol/H2O (v/v), fur-
ther diluted in culture medium, and added to cell cultures as 10-fold 
centrated solutions. Reactions were stopped by the addition of 
100 µl of reconstituted Lucite reagent. Plates were dark-adapted for 
10 min and luciferase expression (luminescent count per second) 
measured using a 12-channel Packard TopCount scintillation 
counter. The amount of luciferase expressed in each well, which is 
proportional to the luminescent count per second measured, was 
expressed as the percentage of response measured in forskolin-
treated control cells. Dose-response curves were fitted by nonlinear 
fit using GraFit version 4.09 to determine EC50 values.

In Vivo Pharmacological Assays

Animals. Male CD-1 mice weighing 25 to 35g and male CD rats 
weighing 220 to 280 g were obtained from Charles River Italia 
(Calo, Italy). Mice and rats were housed in groups of 10 and two, 
respectively, in Plexiglas cages with food and water available ad 
libitum. Animals were maintained at 22 ± 0.5°C with an alternating 
12-h light/dark cycle and were used only once in all experiments.

Hot-Plate Test. Antinociception was assessed utilizing the hot-
plate apparatus (Ugo Basile, Comerio, Italy), maintained at a 
constant temperature of 55 ± 0.1°C, as described by Eddy and Leimbach 
(1953). Briefly, each mouse was placed on the hot-plate and a reac-
tion time measured starting from the placement of the mouse on the 
hot-plate and continuing until the initiation of licking or rapid move-
ment of the hindpaw. Control latencies were approximately 4 to 9 s. 
A test cut-off time of 25 s was chosen to avoid possible tissue damage 
resulting from the test.

Thermal Hyperalgesia. Thermal hyperalgesia was assessed us-
ing the rat plantar test (Ugo Basile), as described by Hargreaves et al. 
(1988). Briefly, hyperalgesia was induced by an intraplantar 
injection of 0.1 ml of a 2% (w/v) suspension of lambda carrageenan 
into the right hindpaw of the animal. Thermal hyperalgesia was 
evaluated 3 h after the carrageenan injection.

Induction of Morphine Tolerance. Mice were treated once 
a day for 4 days with a high dose of morphine hydrochloride (50 mg/kg 
s.c.), as described by Zarrindast et al. (1996). On day 5, 24 h after the 
last administration of morphine, mice were challenged with a lower 
dose of morphine (9 mg/kg s.c.), and the degree of developed tolerance 
was measured by the hot-plate test.

Drug Administration. Nociceptin, lambda carrageenan, and 
morphine hydrochloride were dissolved in 0.9% (w/v) NaCl. SB-
612111A was dissolved in 10% (w/v) encapsin containing 5% (w/v) 
glucose. Intracerebroventricular administration was performed as 
described by Porreca et al. (1984). Mice were lightly anesthetized 
with ether and an incision made in the scalp. The injection was made 
at a point 2 mm caudal and 2 mm lateral from bregma, using a 10-µl 
Hamilton syringe. Compounds were injected at a depth of 3 mm in a 
volume of 5 µl.

Statistical Analysis. Results are presented as mean value ± 
S.E.M. The statistical significance between groups was established 
by analysis of variance followed by Duncan’s multicomparison test. 
P > 0.05 was considered as indicative of significance.

Results

Selective Binding Site Interaction of SB-612111 with 
hORL-1. The affinity and selectivity in vitro of SB-612111 
(structure shown in Fig. 1), and of the corresponding enan-

![Fig. 1. Chemical structure of SB-612111.](image-url)
tioner SB-612112, with human opioid receptors was evaluated in radioreceptor binding assays using membranes prepared from clonal cell lines expressing the human ORL-1 receptor, hMOR, hDOR, and hKOR opioid receptors. SB-612111 and SB-612112 both competitively displaced binding of the ORL-1 receptor radioligand [3H]nociceptin to hORL-1, yielding K_i values in these assays of 0.33 ± 0.03 and 1.3 ± 0.23 nM, respectively (Table 1). Competition studies in assays that measure specific [3H]DAMGO, [3H]DPDPE, and [3H]U-69593 binding to hMOR, hDOR, and hKOR receptors showed that SB-612111 is highly selective versus µ- (174-fold), δ- (6391-fold), and κ (486-fold)-opioid receptors. By comparison, SB-612112 had lower affinity for the hORL-1 receptor and was substantially less selective toward the µ-opioid receptor (27-fold). The δ- and κ-opioid receptor selectivities were, however, similar for both ligands. The data for SB-612111 compare favorably with results obtained in these radioreceptor assays for JTC-801, and the (+)-enantiomer of J-113397, ligands previously described as ORL-1 receptor antagonists. In this study, the observed µ-opioid/ORL-1 receptor selectivities of JTC-801 and (+)-J-113397 were only 3- and 15-fold, respectively, and the κ-opioid/ORL-1 selectivity of (+)-J-113397 was 29-fold. Furthermore, significant separation in the ORL-1 receptor affinity values was observed for the J-113397 enantiomers (79-fold). As expected, the µ-opioid (DAMGO, morphine), δ-opioid (DPDPE), and κ-opioid (BRL 52626, U-69593) ligands and the opioid antagonist naloxone did not compete for [3H]nociceptin binding at concentrations of up to 1 µM, but effectively displaced radioligand binding to the receptors for which they are known to have high affinity, demonstrating the selectively of the assays used.

To assess the specificity by which SB-612111 regulates ORL-1 receptor activity, a broad profiling of its interaction in vitro with receptors from outside of the opioid receptor family, as well as ion channels and enzymes, was undertaken. SB-612111 weakly inhibited activity in several assays (predicted K_i values of <500 nM). These interactions were then investigated in more detail. Affinity (K_i) values were established for competitive displacement of [3H]prazosine binding to the α1A-adrenergic receptor (K_i = 196 nM), [3H]RX 821002 binding to the α2A-adrenergic (K_i = 187 nM) and α2C-adrenergic (K_i = 395 nM) receptors, 125I-cyanopindolol binding [in the presence of 1 µM (+)-propranolol] to the α1A-adrenergic receptor (K_i = 86 nM) and 125I-aminopotentidine binding to the H_2-histamine receptor (K_i = 444 nM).

**Measurement of hORL-1 Receptor Antagonism by SB-612111 in Vitro.** The functional ORL-1 receptor antagonist activity of SB-612111 was evaluated by measuring its ability to antagonize inhibition by nociceptin of forskolin-induced luciferase expression in HEK293 cells stably expressing both hORL-1 and a CRE-luciferase gene reporter. Nociceptin inhibited forskolin-induced luciferase expression in HEK293 cells lines stably expressing both hMOR and a CRE-luciferase gene reporter construct, when over, SB-612111 was able to effectively reverse the inhibitory effect of 0.1 nM nociceptin on luciferase expression (Fig. 2a). Moreover, SB-612111 was able to effectively reverse the inhibitory effect of 0.1 nM nociceptin on luciferase expression in a dose-dependent manner (EC_{50} = 0.009 ± 0.001 nM). SB-612111 produced a dose-dependent rightward shift of this nociceptin response (Fig. 2a). Moreover, SB-612111 was able to effectively reverse the inhibitory effect of 0.1 nM nociceptin on luciferase expression in a dose-dependent manner (K_i = 5 nM), as shown in Fig. 2b. Importantly, SB-612111 did not show any agonist or antagonist activity in HEK293 cells lines stably expressing both hMOR and a CRE-luciferase gene reporter construct, when tested at concentrations of up to 1 µM (data not shown).

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>ORL-1 K_i (nM)</th>
<th>µ K_i (nM)</th>
<th>δ K_i (nM)</th>
<th>κ K_i (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nociceptin</td>
<td>0.079 ± 0.021</td>
<td>128.5 ± 7.5</td>
<td>&gt;2500</td>
<td>49.4 ± 9.3</td>
</tr>
<tr>
<td>SB-612111</td>
<td>0.33 ± 0.03</td>
<td>57.6 ± 8.0</td>
<td>2109 ± 570</td>
<td>160.5 ± 22.4</td>
</tr>
<tr>
<td>SB-612112</td>
<td>1.3 ± 0.23</td>
<td>34.5 ± 5.0</td>
<td>&gt;2500</td>
<td>161 ± 4</td>
</tr>
<tr>
<td>JTC-801</td>
<td>30.8 ± 3.0</td>
<td>93.3 ± 32.1</td>
<td>&gt;2500</td>
<td>1659 ± 400</td>
</tr>
<tr>
<td>(-)-J-113397</td>
<td>157 ± 41</td>
<td>33.7 ± 2.9</td>
<td>&gt;2500</td>
<td>199 ± 16</td>
</tr>
<tr>
<td>(+)-J-113397</td>
<td>2.0 ± 0.7</td>
<td>30.7 ± 7.6</td>
<td>&gt;2500</td>
<td>58.3 ± 6.9</td>
</tr>
<tr>
<td>DAMGO</td>
<td>&gt;10,000</td>
<td>0.58 ± 0.03</td>
<td>1260 ± 195</td>
<td>185 ± 13</td>
</tr>
<tr>
<td>Morphine</td>
<td>&gt;10,000</td>
<td>0.70 ± 0.07</td>
<td>336 ± 102</td>
<td>27.1 ± 4.0</td>
</tr>
<tr>
<td>DPDPE</td>
<td>&gt;5000</td>
<td>200 ± 35.8</td>
<td>6.1 ± 0.6</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>BRL 52626</td>
<td>&gt;10,000</td>
<td>371 ± 32</td>
<td>&gt;2500</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>U-69593</td>
<td>&gt;10,000</td>
<td>510 ± 61</td>
<td>&gt;2500</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>Naloxone</td>
<td>&gt;10,000</td>
<td>0.42 ± 0.05</td>
<td>38.7 ± 9.9</td>
<td>1.21 ± 0.10</td>
</tr>
</tbody>
</table>

**Fig. 2.** In vitro antagonist activity of SB-612111. Activity in the CRE-luciferase gene reporter assay. a, cells were treated with increasing concentrations of nociceptin in the absence (●) or in the presence of 10 nM (○), 30 nM (▲) or 100 nM (△) of SB-612111. b, cells were treated with increasing concentrations of nociceptin (○) or SB-612111 (△), or, varying concentrations of SB-612111 in the presence of 0.01 nM nociceptin (▲). Results are expressed as percentage of forskolin-induced luciferase expression and represent the mean ± S.E.M. from four independent experiments.
were 7.06 ± 0.22 s. Intravenous (i.v.) administration of nociceptin (0.6–10 nmol/mouse) led to a significant reduction in paw withdrawal latencies at the higher doses, as shown in Fig. 3a. Paw withdrawal latencies of nociceptin-treated animals were 2.15 ± 0.35 s versus saline values of 7.06 ± 0.62 s. Intravenous (i.v.) administration of SB-612111 (0.1–3 mg/kg) antagonized the effect elicited by i.c.v. administration of nociceptin (5 nmol) in this test in a dose-dependent manner [ED50 = 0.62 mg/kg, 95% confidence limit (CL) = 0.22–1.89], as shown in Fig. 3b. However, the administration of 1.3 mg/kg i.v. SB-612111 alone did not cause any significant effect on paw withdrawal latencies (data not shown).

The ability of SB-612111 to reverse the nociceptin antagonism of morphine analgesia was also evaluated in the hot-plate test in mice (Fig. 4). Nociceptin (5 nmol/mouse i.c.v.) inhibited morphine (5 mg/kg s.c.)-induced analgesia in the mouse hot-plate test, measured as a reduction in paw withdrawal latencies: morphine administration (5 mg/kg s.c.) produced a withdrawal latency of 21.84 ± 1.07 s, whereas the latency after morphine administration (5 mg/kg s.c.) together with nociceptin (5 nmol i.c.v.) was shortened to 5.55 ± 0.68 s. Administration of SB-612111 i.v. concomitantly with 5 mg/kg s.c. morphine, and 20 min before the administration of 5 nmol/mouse i.c.v. nociceptin, led to a dose-dependent inhibition of the antagonism by nociceptin of the analgesia induced by morphine (ED50 = 0.69 mg/kg i.v., 95% CL = 0.34–1.21), as shown in Fig. 4. Together, these results demonstrate that SB-612111 can act in vivo to antagonize acute nociceptive activity mediated by the ORL-1 receptor.

Reversal of Morphine Tolerance by SB-612111. The ability of SB-612111 to alter pain response in the mouse hot-plate test was further evaluated in mice exhibiting a reduced response (tolerance) to morphine after chronic administration of the drug. Mice were made morphine-tolerant after 4 days of administering 50 mg/kg s.c. morphine once a day. One day after the last morphine dosage animals were challenged with a lower dose of morphine (9 mg/kg s.c.), and analgesic activity was measured on the hot-plate apparatus at 15, 30, 45, and 60 min after drug administration. The peak analgesic activity afforded by the acute morphine administration occurred after 30 min in both drug naïve and chronically treated animals (data not shown). As expected, a reduction in the antinociceptive effect of morphine was observed after chronic morphine administration, which is consistent with the development of opiate tolerance (Fig. 5). In drug-naive mice, the administration of 3 mg/kg i.v. SB-612111 60 min before performing the hot-plate test failed to elicit any significant antinociceptive effect. However, the administration of SB-612111 to animals chronically treated with morphine produced a marked enhancement in the analgesic effect of an acute dose (9 mg/kg s.c.) of morphine. Together, these results demonstrate that an ORL-1 receptor antagonist can act to resensitize animals to the analgesic effect of morphine after chronic opiate exposure. It should be further noted that although the potential analgesic activity of SB-612111 per se in morphine-tolerant mice was not tested...
SB-612111 is a member of a novel class of nonpeptide antagonists that is structurally unrelated to other nonpeptide ORL-1 receptor ligands that have been described, such as J-113397 (Kawamoto et al., 1999) and JTC-801 (Shinkai et al., 2000).

Profiling of SB-612111 in competition radioligand binding experiments indicated some preference of the (+)-enantiomer (SB-612111) for the ORL-1 receptor and a higher selectivity versus the μ-opioid receptor, and all further studies were carried out using SB-612111. SB-612111 proved in these tests to have both higher affinity for the ORL-1 receptor and greater selectivity versus the μ-opioid receptor than the other ORL-1 antagonists tested (JTC-801 and J-113397). Interestingly, the resolved enantiomers of J-113397 differed considerably in their affinity for the ORL-1 receptor, with the (+)-enantiomer having 79-fold higher affinity than that measured for the (−)-enantiomer. Extended profiling of SB-612111 in assays specific for a variety of receptors, ion channels, and enzymes revealed several weak interactions that were explored in more detail. However, the highest measured affinity interaction of SB-612111, to the β2-adrenergic receptor, was >260-fold weaker than its interaction with the ORL-1 receptor. Cellular signaling assays further established that SB-612111 is an ORL-1 receptor antagonist with low potential for either activation or antagonism of the μ-opioid receptor. In summary, these data suggest that SB-612111 would allow us to examine ORL-1 receptor function in vivo.

We established that intracerebroventricular injection of nociceptin dose dependently shortens hot-plate latencies in mice. These results are in agreement with behavioral studies demonstrating that i.c.v. injection of low doses of nociceptin produces a hyperalgesic response in mice, as measured by the hot-plate or tail-flick test (Meunier et al., 1995, Reinscheid et al., 1995). Intravenous injection of SB-612111 dose dependently inhibited acute pain signaling induced by i.c.v. nociceptin in the mouse hot-plate assay without altering baseline hot-plate latencies. These results demonstrated that SB-612111 acts as an ORL-1 receptor antagonist in vivo as well as in vitro. The lack of antinociceptive or hyperalgesic effects of SB-612111 per se in the mouse hot-plate test suggests that ORL-1 receptor activation does not directly modulate acute nociceptive signaling. Earlier results by Yamada et al. (2002) demonstrating a potent effect of JT-801 alone in

**Fig. 5.** Sensitization of response after SB-61211 treatment of morphine-insensitive mice. SB-612111 (3 mg/kg i.v.) and morphine (9 mg/kg s.c.) were administered 60 and 30 min before performing the hot-plate test, respectively. Experiments with tolerant animals were performed 24 h after the final high-dose (50 mg/kg s.c., once a day) of morphine was administered and 5 days after initiating the chronic opiate dosing. Each value shown represents the mean ± S.E.M. for 10 mice. *, $P < 0.05$ compared with the vehicle + saline-treated group. #, $P < 0.05$ compared with the vehicle + morphine-treated group.

**Fig. 6.** Antihyperalgesic activity of SB-612111 in the rat carrageenan plantar test. Paw withdrawal latencies are shown for the ipsilateral (filled column) and contralateral paw (open column). Each value represents the mean ± S.E.M. for eight rats. Paw withdrawal latencies for saline-treated rats were 11.05 ± 0.50 s (ipsilateral) and 10.97 ± 0.79 s (contralateral), whereas for carrageenan-treated rats these values were 4.00 ± 0.50 s (ipsilateral) and 11.22 ± 0.51 s (contralateral). *, $P < 0.05$ compared with vehicle + carrageenan-treated group.

In this study, Ueda et al. (2000) reported earlier that the ORL-1 antagonist J-113397 does not alter response in tail pinch or tail-flick analgesia tests using morphine-tolerant mice.

**Antihyperalgesic Activity of SB-612111.** To test a suspected role of the ORL-1 receptor in persistent pain responses, we used the carrageenan plantar model of inflammatory pain to evaluate the potential antihyperalgesic effect of SB-612111. After injection of carrageenan into the rat hindpaw, a painful inflammatory response develops that can be measured by a shortening in ipsilateral paw withdrawal latencies to a noxious stimulus. Paw withdrawal latencies in the carrageenan-treated animals were 4.00 ± 0.50 s versus 11.05 ± 0.52 s in saline-treated animals. In this model, SB-612111 caused a significant inhibition of the carrageenan-induced reduction in paw withdrawal latencies at doses of 3 and 5 mg/kg i.v., whereas paw withdrawal latencies in the contralateral, untreated paw were unaffected (Fig. 6).

**Discussion**

The availability of the high-affinity and selective antagonist SB-612111 has allowed us to probe the role of the ORL-1 receptor in regulating nociception. SB-612111 is a member of a novel class of nonpeptide antagonists that is structurally unrelated to other nonpeptide ORL-1 receptor ligands that have been described, such as J-113397 (Kawamoto et al., 1999) and JTC-801 (Shinkai et al., 2000).
this test may be explained by a possible interaction with 
\(\mu\)-opioid receptors, because our in vitro data (summarized in 
Table 1) revealed a low \(\mu\)-opioid/ORL-1 receptor selectivity 
for this compound. Our data also agree with studies showing 
that J-113397 can inhibit nociceptin-induced hyperalgesia in 
the mouse tail-flick assay without altering baseline tail-flick 
latencies (Ozaki et al., 2000) and that ORL-1 receptor knock- 
out mice display the same nociceptive threshold as control 
mice in acute pain models (Nishi et al., 1997). These earlier 
findings have suggested, and our data agree with, the hy-
pothesis that the nociceptin/ORL-1 system is activated in 
evoked pain states. SB-612111 likely has a central site of 
action in these studies, as evidenced by its ability to antag-
onize the action of centrally administered nociceptin. How-
ever, its action at peripheral ORL-1 receptors to regulate 
thermal nociception cannot be ruled out, as previously dem-
onstrated by Ko et al. (2002).

In the present study, we additionally demonstrated that 
SB-612111 behaves as an ORL-1 antagonist in vivo by dose 
dependently reversing an inhibition of morphine analgesia 
promoted by central i.c.v. administration of nociceptin. The 
ability of SB-612111 to counter the effects of centrally ad-
ministered nociceptin on morphine responsiveness is consis-
tent with a mechanism in which both \(\mu\)-opioid and ORL-1 
receptor activation influences the release of neurotransmit-
ters such as GABA in descending pain pathways, as de-
scribed by Vaughan et al. (2001). The similar ability of the 
\(\mu\)-opioid and ORL-1 receptors to regulate GABA release, yet 
opposing effects on nociceptive signaling, has been suggested 
to result from differential on- and off-cell expression and 
activity of these receptors in the rostral ventromedial me-
dulla. An alternative view, which builds upon the observa-
tion of \(\mu\)-opioid and ORL-1 receptor coexpression on the same 
cells, uses evidence of receptor cross talk and heterologous 
receptor desensitization to explain the opposing actions of 
\(\mu\)-opioid and ORL-1 receptor activation. For instance, 
Mandyam et al. (2002) have shown that ORL-1 receptor 
activation can cause desensitization of \(\mu\)-opioid receptors 
expressed on the same cell by stimulating translocation of 
protein kinase C isozymes to the cell membrane. Thus, an 
ORL-1 antagonist would be expected to prevent desensitiza-
tion of the \(\mu\)-opioid receptor resulting from nociceptin-medi-
ated ORL-1 receptor activation, and thereby potentiate the 
action of morphine.

We further extended the morphine analgesia studies to 
include tests in mice treated chronically with morphine, and 
now show that a single administration of SB-612111 reverses 
established tolerance to the analgesic effects of morphine in 
the mouse hot-plate test. These data support the hypothesis, 
proposed by Nishi et al. (1997), that changes in neuronal 
plasticity observed in conditions of morphine tolerance may 
derive from increased activity of an antinociceptive nociceptin 
system. Ueda et al. (2000) had previously reported the re-
tarded acquisition of morphine tolerance in ORL-1 receptor 
knockout mice, and its reversal in mice after a single subcun-
table or intrathecal administration of J-113397. Thus, it 
will be of further interest to establish a linkage of the ORL-1 
receptor to signaling pathway elements that can regulate 
\(\mu\)-opioid receptor function. One such key signaling rela-
tionship may be with RGS4, a member of the regulator of G 
protein signaling (RGS) protein family. Recently, we reported 
a relationship between morphine tolerance and the expres-
sion of RGS4 and further established that this signal-regu-
ulating protein can influence \(\mu\)-opioid receptor function (Gar-
nier et al., 2003).

Previous work by Andoh et al. (1997) showed that periph-
eral inflammation induces nociceptin expression in primary 
sensory neurons and that the ORL-1 receptor may be asso-
ciated with the production of nociceptive hypersensitivity 
associated with an inflammatory response. Our results, 
showing that SB-612111 had antihyperalgesic activity in the 
subchronic, rat carrageenan inflammatory pain model, are in 
line with this hypothesis. However, these data contrast with 
previous work by Yamamoto et al. (1997), showing that in-
trathecal injection of nociceptin reduces allostynia and ther-
mal hyperalgesia induced by carrageenan injection into the 
rat paw. These discrepancies may be explained by the dose 
dependence of pro- versus antinociceptive activities of noci-
ceptin, as recently described by Muratani et al. (2002) using 
the mouse formalin model of acute pain response. Our data 
showing that SB-612111 can reverse carrageenan-induced 
hyperalgesia are consistent with a role of nociceptin in the 
negative regulation of excitatory neurotransmission. The re-
cent description of a role of the glutamate pathway in this 
hyperlgesia model (Inoue et al., 2003) is consistent with this 
view. However, our results do not suggest a pain-inhibitory 
action of nociceptin via its effects on substance P-ergic fibers 
in the spinal cord, as was also proposed in this report.

In this study, we have described the pharmacological char-
acteristics of SB-612111, a member of a new class of selective, 
nonpeptidic ORL-1 receptor antagonists. We have further 
used this ligand to probe the role of the ORL-1 receptor in 
nociceptive signaling. The data demonstrate that SB-612111 
can effectively antagonize the pronociceptive action of noci-
ceptin in an acute pain model and potentiate the action of 
morphine in animals that are morphine-tolerant. In addition, 
SB-612111 proved to be effective alone in blocking hyperal-
gesia in an inflammatory pain model. Together, the results 
suggest that this novel class of selective ORL-1 antagonist 
may have therapeutic utility in the treatment of evoked pain 
responses such as those occurring in response to inflamma-
tion and in the prolongation of opiate analgesic therapy.

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