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The benzomorphan-based LP1 ligand is a suitable MOR/DOR agonist for chronic pain treatment

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ABSTRACT

Aims: Powerful analgesics relieve pain primarily through activating mu opioid receptor (MOR), but the long-term use of MOR agonists, such as morphine, is limited by the rapid development of tolerance. Recently, it has been observed that simultaneous stimulation of the delta opioid receptor (DOR) and MOR limits the incidence of tolerance induced by MOR agonists. 3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-phenylpropanamide (LP1) is a centrally acting agent with antinociceptive activity comparable to morphine and is able to bind and activate MOR and DOR. The aim of this work was to evaluate and compare the induction of tolerance to antinociceptive effects from treatment with LP1 and morphine.
Main methods: Here, we evaluated the pharmacological effects of LP1 administered at a dose of 4 mg/kg subcutaneously (s.c.) twice per day for 9 days to male Sprague–Dawley rats. In addition, the LP1 mechanism of action was assessed by measurement of LP1-induced [^{35}S]GTPγS binding to the MOR and DOR.
Key findings: Data obtained from the radiant heat tail flick test showed that LP1 maintained its antinociceptive profile until the ninth day, while tolerance to morphine (10 mg/kg s.c. twice per day) was observed on day 3. Moreover, LP1 significantly enhanced [^{35}S]GTPγS binding in the membranes of HEK293 cells expressing either the MOR or the DOR.
Significance: LP1 is a novel analgesic agent for chronic pain treatment, and its low tolerance-inducing capability may be correlated with its ability to bind both the MOR and DOR.

Introduction

Opioid analgesics, such as morphine, are the standard of care for moderate-to-severe pain (McQuay, 1999). However, their long-term use for chronic pain results in the development of tolerance (Ueda and Ueda, 2009), a phenomenon, probably more pronounced in rodents compared to humans (Milne et al., 1996), characterised by a decrease or loss of effect following repeated treatments. Thus, a higher dose is required to achieve a consistent therapeutic effect (Bailey and Connor, 2005). Prevention of tolerance is a critical issue in pain management. Numerous studies have ascertained that the ability of certain opioids to induce tolerance may be related to their efficacy for the mu opioid receptor (MOR) (Alvarez et al., 2002; Ingram and Traynor, 2009), whose distribution is consistent with its role in pain responses (Commons et al., 2000). However, the dissociation of analgesia from tolerance using MOR selective agonists is nearly impossible. Indeed, investigations using MOR knockout mice have demonstrated that both the antinociception and tolerance effects are MOR-mediated (Kieffer and Gavériaux-Ruff, 2002). Conversely, delta opioid receptor (DOR) and kappa opioid receptor (KOR) selective agonists induce less tolerance than MOR agonists; however, these compounds induce only weak antinociception. Moreover, the design and synthesis of ligands highly selective for DOR and KOR as a strategy to overcome or limit MOR-mediated tolerance were unsuccessful (Eguchi, 2004; Bodnar, 2010). Compounds possessing multitarget opioid activity are effectively antinociceptive with limited adverse effects (Prezzavento et al., 2010; Dietis et al., 2009). Recently, the in vivo co-administration of selective MOR and DOR agonists showed synergic antinociceptive activity with less induced tolerance (Negus et al., 2009). Furthermore, biochemical studies have demonstrated that prolonged stimulation of MOR induces DOR trafficking to plasma membrane, supporting the existence of physical and functional modulatory interactions between the MOR and DOR (Zhang and Pan, 2010). Consequently, a bifunctional ligand interacting with both MOR and DOR could be a useful drug for the treatment of chronic pain. Bifunctional ligands may have better compliance and a lesser incidence of drug interactions (Morphy and Rankovic, 2005).

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We recently described the synthesis and structure–activity relationships of a new series of opioid ligands (Pasquinucci et al., 2010) based on the 6,7-benzomorphan class (Ronsisvalle et al., 1993, 1995). From our screening analysis, we developed 3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-phenylpropanamide, termed LP1 (Fig. 1), which exhibited high and moderate affinity for MOR and DOR, respectively ($K_i^{MOR} = 0.83 \pm 0.05$ nM, $K_i^{DOR} = 29 \pm 1$ nM). Moreover, LP1 acts as a MOR/DOR agonist as assessed by intracellular cAMP accumulation ($IC_{50}^{MOR} = 4.8 \pm 0.5$ nM and $IC_{50}^{DOR} = 12 \pm 1.2$ nM) and has similar antinociceptive potency to morphine ($ED_{50} = 2.03$ mg/kg s.c. vs. 2.7 mg/kg s.c.) as assessed by the radiant heat tail-flick test. In light of the significant antinociceptive effect after acute administration of LP1 and its MOR/DOR agonistic profile, the purpose of the present study was to assess and compare the induction of tolerance to the antinociceptive effects of LP1 and morphine. We also evaluated the LP1-induced activation of G proteins to define a potential mechanism of action that may describe the delayed onset of tolerance (Weiland and Jakobs, 1994).

Materials and methods

Animals

Male Sprague–Dawley rats (Morini, S. Polo d'Enza, RE, Italy) weighing 180–200 g were used. Animals were kept at a constant room temperature (25 ± 1 °C) under a 12:12 h light and dark cycle with free access to food and water. Each rat was used for only one experiment. Experimental procedures were approved by the local ethical committee (IACUC) and were conducted in accordance with international guidelines, such as those of the European Community, and national regulations (CEE Council 86/609 and DL 116/92).

Nociceptive test

Nociception was evaluated by the radiant heat tail-flick test (Scoto et al., 2010). Briefly, this assay consists of the irradiation of the lower third of the tail with an I.R. source (Ugo Basile, Comerio, VA, Italy). The day before the experiment, rats were habituated to the procedure, and the nociception threshold was measured. The basal pre-drug latency was between 3 and 4 s, which was calculated from the average of the first three measurements performed at 5 min intervals. A cut-off latency of 10 s was established to minimise damage to the tail. The behavioural tests were conducted by researchers blinded to the treatment group. For assessment of tolerance, rats were divided into three groups (each consisting of 8–10 animals). Rats received morphine hydrochloride (10 mg/kg s.c.), LP1 (4 mg/kg s.c.) or saline (control) s.c. twice a day (9.30 a.m. and 3.30 p.m.) and post-treatment tail-flick latencies (TFLs) were determined 30 min after the morning subcutaneous (s.c.) injection. A decrease in antinociceptive response was considered indicative of tolerance development. Data are expressed as the mean ± S.D. of each group. The means for each group were analysed by two-way or one-way ANOVA, where appropriate, followed by Student–Newman–Keuls test. Differences were considered significant when $p < 0.05$.

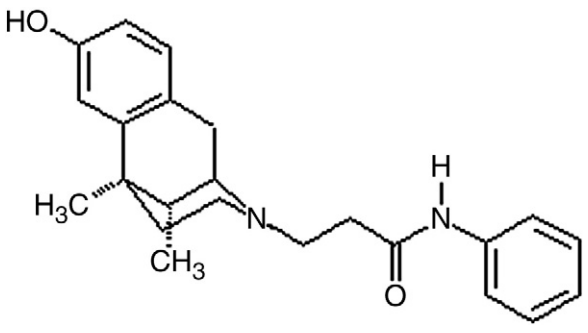

Fig. 1. Structure of the benzomorphan-based opioid ligand LP1.

Cell culture and membrane preparation

Human embryonic kidney (HEK293) cells stably expressing either the MOR or the DOR were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37 °C under a 5% CO_2 atmosphere as previously described (Morou and Georgoussi, 2005; Leontiadis et al., 2009). Confluent monolayers of HEK293 cells stably expressing the MOR or the DOR were harvested, collected by centrifugation at 1500 rpm for 5 min and washed once with phosphate-buffered saline (PBS) (pH 7.5). Cell membranes were prepared as described by Georgoussi and Zioudrou (1993). Briefly, cell pellets were resuspended in ice-cold membrane buffer A (10 mM Tris–HCl, pH 7.5, and 0.1 mM EDTA), homogenised and centrifuged at 2000 rpm for 3 min at 4 °C. Supernatants were further centrifuged at 45,000 rpm for 30 min at 4 °C. The membrane pellet was resuspended in ice-cold buffer A at a protein concentration of approximately 1 mg/ml and stored in aliquots at −70 °C. The protein concentration was determined according to the method of Bradford (1976).

[^{35}S]GTPγS binding studies

[^{35}S]GTPγS binding was performed on membranes from HEK293 cells stably expressing either the MOR or the DOR as described by Georgoussi et al. (1997). Membranes expressing the MOR (7.5 μg of protein per reaction) or the DOR (12 μg of protein per reaction) were added to a reaction mixture (100 μl) containing 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, 0.2 mM ascorbate, 0.3–0.5 nM [^{35}S] GTPγS (50 nCi), and the appropriate ligand (0.1 nM–10 μM) and were incubated for 60 min at 30 °C or 4 °C for MOR and DOR, respectively. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. The reaction was terminated by rapid filtration through GF/C Whatman filters followed by three washes with 4 ml of ice-cold 20 mM HEPES (pH 7.4) containing 3 mM $MgCl_2$ using a Brandel cell harvester. Bound radioactivity was measured by liquid scintillation counting (Liquid Scintillation Analyzer, Packard). Analysis of the binding data was performed using Origin 7.5 software (OriginLab Corporation, Northampton, USA). Data represent the percent of ligand-induced [^{35}S]GTPγS binding over basal activity, defined as [(specific binding/basal binding) × 100] − 100. Experiments were repeated at least three times and were performed in duplicate. To determine the antagonist activity of LP1 at the MOR, HEK293 cells expressing MOR were incubated with LP1 in presence of 100 nM of the MOR agonist DAMGO. Similarly, to determine antagonist activity of LP1 at the DOR, HEK293 cells expressing DOR were incubated with LP1 in presence of 100 nM of the DOR agonist DPDPE. Statistical analysis was performed with one-way analysis of variance and Student's *t*-test, with $p < 0.05$ indicating significance.

Drugs

LP1 was synthesised as previously reported (Pasquinucci et al., 2010); morphine hydrochloride was purchased from S.A.L.A.R.S. (Como, Italy); DAMGO, DPDPE, GTP and all other reagents were of analytical grade from Sigma-Aldrich; [^{35}S]GTPγS (1250 Ci/mmol) was obtained from PerkinElmer; reagents for tissue culture were from Gibco and Invitrogen. For in vivo studies all drugs were dissolved in 0.9% sterile saline. For in vitro assay drugs were dissolved in H_2O, where necessary ethanol was used.