Antinociceptive profile of LP1, a non-peptide multitarget opioid ligand

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Aims: Opioid drugs are the principal treatment option for moderate to severe pain and exert their biological effects through interactions with opioid receptors that are widely distributed throughout the CNS and peripheral tissues. Ligands capable of simultaneously targeting different receptors could be successful candidates for the treatment of chronic pain. Enhanced antinociception coupled with a low incidence of side effects has been demonstrated for ligands possessing mixed mu-opioid receptor (MOR) and delta-opioid receptor (DOR) activity. We previously reported that 3-[(2R,6R,11R)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2H)-yl]-N-phenylpropanamide (LP1) acted as a MOR-DOR ligand in in vitro functional assays and moreover this drug produced a valid antinociception that was longer lasting than that of morphine. The aim of this work was to determine whether the antinociceptive effect produced by LP1 was central or peripheral and to assess which opioid receptor subtypes are involved in its effects.

Main methods: We explored the effects of naloxone methiodide (NX-M), a quaternary opioid antagonist, administered either intracerebroventricularly (i.c.v.) or subcutaneously (s.c.), on LP1-mediated antinociception. In addition, we administered s.c. selective antagonists for MOR, DOR and kappa-opioid receptor (KOR) to investigate the effects of LP1. To characterise this drug’s DOR profile better, we also investigated the effects of LP1 on DPDPE, a selective DOR agonist.

Key findings: Data obtained by tail flick test showed that LP1 induced predominantly MOR-mediated supraspinal antinociception and was able to counteract DPDPE analgesia.

Significance: LP1, a multitarget opioid ligand, is a supraspinal acting antinociceptive agent that is useful for the treatment of chronic pain.

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Introduction

Moderate or severe pain is managed mainly with opioid analgesics (Power, 2011), the pharmacological effects of which are derived from their interactions with the mu-opioid receptor (MOR), delta-opioid receptor (DOR) or kappa-opioid receptor (KOR) (Dietis et al., 2011). These receptors are found in the periphery at the presynaptic and postsynaptic level, in the spinal cord dorsal horn and in the brainstem, thalamus and cortex, where they represent an inhibitory system for the ascending and descending modulation of pain transmission (Inturrisi, 2002). Despite their well-established clinical utility, the long-term use of opioids is limited by wide variety of side effects, including tolerance development (Ueda and Ueda, 2009). Ligands capable of simultaneously targeting different receptors could be successful candidates for the treatment of chronic pain (Dietis et al., 2009; Prezzavento et al., 2010; Schiller, 2010). Indeed, an improved antinociception and a low propensity to tolerance development were reported for ligands possessing mixed MOR–DOR opioid activity. These pharmacological effects have been described for the DOR antagonist–MOR agonist peptidic ligands KSK-103 and UFP-505 (Purington et al., 2011; Balboni et al., 2010), as well as for the DOR antagonist peptidic ligands MMP2200 and BVD03 (Lowery et al., 2011; Vandormael et al., 2011). We previously identified a compound based on the 6,7-benzomorphan structure, 3-[(2R,6R,11R)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2H)-yl]-N-phenylpropanamide (LP1, Fig. 1), that showed an interesting pharmacological profile, as assessed by in vitro and in vivo studies (Pasquinucci et al., 2010). In particular, LP1 exhibited MOR and DOR affinity and intrinsic activity in binding studies and in vitro functional assays, respectively. Concerning its antinociceptive effect, our compound was comparable to morphine however with a lower tolerance-inducing capability (Pasquinucci et al., 2012). Building upon our previous results, the present study reports the effects of naloxone methiodide (NX-M), a non-selective opioid receptor antagonist that is unable to cross the blood brain barrier (BBB), by intracerebroventricular (i.c.v.) or subcutaneous (s.c.) injection, to determine whether the antinociception produced by LP1 is central or peripheral (Craft et al., 1995). Moreover, to assess the involvement of the different opioid receptor subtypes in the antinociceptive effect of LP1, we used the selective MOR, DOR
and KOR antagonists naloxonazine (NLZ), naltrindole (NTI) and norbinaltorphimine (norBNI), respectively, each of which has unrestricted access to both the central and peripheral nervous system (Bedini et al., 2010).

Materials and methods

Animals

Experiments were conducted on male Sprague–Dawley rats (Harlan, San Pietro al Natisone (UD), Italy) weighing 180–200 g. The animals were kept in cages 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. On three consecutive days prior to behavioural testing, rats were handled regularly and gradually habituated to the testing equipment. To minimise animal anxiety, all tests were performed between 09:00 and 15:00 in a quiet, isolated room. The behavioural tests were conducted by researchers blinded to the treatment group. Experimental procedures were approved by the local ethical committee and the Institutional Animal Care and Use Committee (IACUC), and all experiments were conducted in accordance with International Guidelines and the European Communities Council Directive and National Regulations (EEC Council 86/609 and DL 116/92).

Surgery

Rats were anaesthetised with Zoletil® 50 mg/kg (tiletamine chloridate 125.0 mg and zolazepam chloridate 125.0 mg) in the quadriiceps muscle and were mounted on a stereotaxic instrument equipped with a homeothermic controller pad. The skull was exposed, and a stainless steel guide cannula (21 Ga; Plastic One, Roanoke, VA, USA) was inserted directly into the lateral ventricle (1.5 mm from the sagittal suture, 1.5 mm posterior to the bregma and at a depth of 4.0 mm from the skull, as recommended by Paxinos and Watson (2007)) and fixed to the skull with acrylic dental cement. The experiments began 5 days after recovery. A microinjection system (Kd Scientifics/Biological Instruments) was used to inject the drugs into the ventricle. A stainless steel needle (26 Ga and 5.5 mm; Plastic One, Roanoke, VA, USA) was attached to PE20 portex tubing and placed into the implanted cannula. The injection volume of each drug was 5 μl followed by 5 μl saline to flush out any drug residue. The needle remained in place for 20 s after infusion. At the end of the experiments, the microinjection site was marked by injecting 5 μl cresyl violet into the ventricle, and the rats were sacrificed under deep anaesthetia. The injection location was determined in histological sections based on the Paxinos and Watson (2007). Data from animals with improperly placed cannulae were discarded (Scoto et al., 2005).

Nociceptive test

Nociception was evaluated by the radiant heat tail-flick test, as previously reported (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. Post-treatment tail-flick latencies (TFLs) were determined at 20, 40, 60, 80 and 100 min after injection.

Experimental procedure

The rats were divided into the following 10 groups (each consisting of 8–10 animals) for the tail-flick test experiment:

- Group 1 (control animals): saline s.c.;
- Group 2 LP1 (3 mg/kg s.c.);
- Group 3 NX-M (3 mg/kg s.c.) followed by LP1 (3 mg/kg s.c.) after 30 min;
- Group 4 NX-M (5 μg/5 μl i.c.v.) followed by LP1 (3 mg/kg s.c.) after 5 min;
- Group 5 NLZ (35 mg/kg s.c.) followed by LP1 (3 mg/kg s.c.) after 24 h;
- Group 6 nor-BNI (10 mg/kg s.c.) followed by LP1 (3 mg/kg s.c.) after 30 min;
- Group 7 NTI (1 mg/kg s.c.) followed by LP1 (3 mg/kg s.c.) after 30 min;
- Group 8 DPDPE (20 μg/5 μl i.c.v.);
- Group 9 NLZ (35 mg/kg s.c.) followed by DPDPE (20 μg/5 μl i.c.v.) after 24 h;
- Group 10 NLZ (35 mg/kg s.c.) followed by LP1 (3 mg/kg s.c.) after 24 h, followed by DPDPE (20 μg/5 μl i.c.v.) after 5 min.

Drugs

LP1 was synthesised as previously reported (Pasquinucci et al., 2010); nor-binaltorphimine dihydrochloride (nor-BNI), naltrindole hydrochloride (NTI), naloxonazine dihydrochloride (NLZ) and DPDPE were purchased from Tocris (Bristol, UK); naloxone methiodide (NX-M) was purchased from Sigma-Aldrich and Zoletil® was bought from Farmavet (Catania, Italy). All drugs were dissolved in 0.9% sterile saline.

Statistical analysis

The data were expressed as means ± S.E.M. To assess overall effect, areas under the time course curves (AUC) of the thermal withdrawal thresholds were calculated using the trapezoidal method. Intergroup comparisons were assessed using an initial two-way analysis of variance (ANOVA), followed by Duncan’s multiple range test. Differences were considered significant when p < 0.05.

Results

LP1, at a dose of 3 mg/kg s.c., caused a significant increase in tail flick latencies with respect to the saline control group (6.4 ± 0.5 s, 9.1 ± 0.8 s, 7.2 ± 0.7 s, 6.8 ± 1.5 s at 20, 40, 60 and 80 min after treatment, respectively) (Fig. 2, panel A). The i.c.v. administration of 5 μg/5 μl of the antagonist NX-M, a quaternary derivative that does not readily cross the blood–brain barrier (BBB), at 5 min before LP1 significantly reduced the antinociceptive effect of the compound (5.3 ± 1.8 s, 4.4 ± 2.3 s and 3.6 ± 0.7 s at 40, 60 and 80 min of observation, respectively); these results were significantly different from the values obtained after LP1 injection at the same timepoints (Fig. 2, panel A). The tail flick latencies were not modified by the s.c. injection of 3 mg/kg NX-M administered 30 min before LP1 (Fig. 2, panel A). The obtained results are well highlighted by the AUC estimation (Fig. 2, panel A). To evaluate the involvement of the different opioid receptor subtypes in LP1-induced antinociception, pre-treatment with selective MOR, DOR and KOR opioid antagonists were performed. NLZ, the selective MOR antagonist, at
the dose of 35 mg/kg s.c., completely antagonised LP1 antinociception when administered 24 h prior to LP1 (the values recorded were significantly different from those in LP1-treated rats at 40, 60 and 80 min of observation: 2.3 ± 0.5 s, 2.6 ± 0.4 s and 2.8 ± 0.3 s) (Fig. 3, panel A). The expression of the values as AUC confirmed the inhibition of LP1 antinociceptive effect (Fig. 3, panel A1). A 10 mg/kg s.c. dose of the selective KOR antagonist norBNI partially blocked the antinociceptive effect of our compound when administered 30 min before LP1 (time course values: 6.6 ± 1.5 s, 4.5 ± 0.8 s and 4.5 ± 1.5 s at 40, 60 and 80 min after treatment, respectively; AUC value: 502.07 ± 59.2 s) (Fig. 4, panel A and A1). In contrast, 1 mg/kg s.c. of the selective DOR antagonist NTI did not significantly alter the antinociception of LP1 when administered 30 min before LP1 (Fig. 5, panel A and A1). The selective opioid antagonists did not change the basal tail flick latencies when administered on their own (data not shown). To explore the putative DOR profile of LP1, we measured the drug’s ability to modify the antinociceptive effect of DPDPE, which is a selective DOR agonist. A dose of 20 μg/5 μl i.c.v. DPDPE caused a significant increase in TFL at 20 min of observation (7.1 ± 1.4 s) (Fig. 6, panel A). NLZ (35 mg/kg s.c.) administered 24 h prior to DPDPE did not change the antinociceptive effect of the compound. In NLZ pretreated-rats, LP1 (3 mg/kg s.c.) administration prior to DPDPE significantly reduced the antinociceptive effect of the peptide (3.9 ± 0.9 s at 20 min of observation) (Fig. 6, panel A). When the results were expressed as AUC the registered value was 372.3 ± 19.5 s (Fig. 6, panel A1).

**Discussion**

In our experiments, systemically administered LP1 showed a supraspinally mediated antinociceptive effect. The LP1-evoked antinociception was not affected in rats pre-treated with s.c. NX-M, a quaternary antagonist that is unable to cross the BBB. Conversely, i.c.v. NX-M pre-treatment led to a significant decrease in the LP1 antinociceptive effect, demonstrating that our compound acts predominantly in the CNS. The supraspinal action of s.c.-injected LP1 could be related to its non-peptidic structure, which is associated with a favourable lipophilicity (aLogP = 3.79) (Tetko et al., 2005; Lee et al., 2011). Unlike most opioid peptides, which are characterised by poor bioavailability after systemic administration (Van Dorpe et al., 2011), LP1 should be able to cross the BBB and target opioid receptors that are widely distributed at the central level (Argoff, 2011) in amounts sufficient to produce antinociception without significantly affecting behavioural responses (i.e. locomotor activity and sedation; unpublished data). The selective MOR antagonist NLZ completely abolished the antinociceptive activity of LP1, clearly and unequivocally indicating the involvement of MOR in the effect of our benzomorphan compound. This finding is consistent with the distribution of MOR throughout the CNS in key sites for the modulation of pain transmission (Arvidsson et al., 1995; Mansour et al., 1995). The antinociception induced by LP1 was attenuated by the KOR antagonist norBNI. In previous competition binding studies, our benzomorphan compound exhibited high and moderate affinities for
MOR ($K_i = 0.83 \pm 0.05 \text{ nM}$) and DOR ($K_i = 29.1 \pm 1 \text{ nM}$), respectively, but a low affinity for KOR ($K_i = 110 \pm 1 \text{ nM}$). Thus, KOR involvement in the antinociception of LP1 could be either the consequence of a direct activation of KOR or an indirect phenomenon that occurs due to MOR stimulation. Data in the literature suggest that the antinociceptive effect of endomorphin-2, a known MOR agonist peptide with no significant affinity for KOR, is attenuated by norBNI pre-treatment (Horvath, 2000; Ohsawa et al., 2001). It has also been demonstrated that MOR stimulation induces the release of endogenous dynorphins that act on KOR to elicit antinociception (Mizoguchi et al., 2006a, b). In contrast, LP1-mediated antinociception was not significantly modified by NTI, the DOR selective antagonist. However, in rats pre-treated with NLZ, s.c. LP1 was able to attenuate significantly the antinociceptive effect induced by i.c.v. injection of the DOR agonist DPDPE. Thus, these data seem to suggest that LP1 is a good MOR agonist and is also able to counteract DPDPE-mediated analgesia. Previously, cAMP accumulation and $[^{35}\text{S}]$GTPγS binding assays performed in cells transfected with either MOR or DOR identified LP1 as a MOR–DOR partial agonist, although

Fig. 4. Effect of nor-binaltorphimine (nor-BNI) (10 mg/kg s.c.) on LP1 (3 mg/kg s.c.) antinociception. Data are expressed (panel A) by the time-course curve of mean ± S.E.M. tail flick latencies (s) and (panel A1) by the mean ± S.E.M. area under the time-course curves (AUC) of tail flick latencies (s), calculated by the trapezoidal method ($n=8$–$10$). *$P<0.05$ vs saline-treated rats. #$P<0.05$ vs LP1-treated rats.

Fig. 5. Effect of naltrindole (NTI) (1 mg/kg s.c.) on LP1 (3 mg/kg s.c.) antinociception. Data are expressed (panel A) by the time-course curve of mean ± S.E.M. tail flick latencies (s) and (panel A1) by the mean ± S.E.M. area under the time-course curves (AUC) of tail flick latencies (s), calculated by the trapezoidal method ($n=8$–$10$). *$P<0.05$ vs saline-treated rats.

Fig. 6. Effect of LP1 (3 mg/kg s.c.) on DPDPE (20 μg/5 μl/rat i.c.v.) antinociception in rats pre-treated with naloxonazine (NLZ) (35 mg/kg s.c.). Data are expressed (panel A) by the time-course curve of mean ± S.E.M. tail flick latencies (s) and (panel A1) by the mean ± S.E.M. area under the time-course curves (AUC) of tail flick latencies (s), calculated by the trapezoidal method ($n=8$–$10$). *$P<0.05$ vs saline-treated rats. #$P<0.05$ vs saline-treated rats. +$P<0.05$ vs DPDPE-treated rats.
with different effects on each receptor (Pasquinucci et al., 2010, 2012). The degree of partial agonism may depend on the relationships between and ratios of receptors and G proteins in the studied system (Berger et al., 2000). Berger et al. (2000) explained that the antagonist, partial agonist or even full agonist properties of F[\gamma\text{CJNC} (1–13)]NH₂ and Ac-EYYRIK-NH₂ on the NOP receptor depended on the evaluated system. Analogously, the in vivo antagonist properties of the DOR ligand MSF61 are associated with partial agonism in vitro (Negus et al., 2009). Therefore, the in vivo DOR profile of LP1 would not be inconsistent with the DOR partial agonist detected in vivo. Differences between in vitro and in vivo functional data are especially common for multitarget ligands. For instance, despite having MOR agonist-DOR antagonist effects detected in vivo, SoR8409 was not able to stimulate MOR [S]GTP-\(\gamma\)S binding (Xu et al., 2001) in vitro, and the in vivo MOR effects of tramadol were not supported by in vitro data (Gillen et al., 2000; Raffa and Friderichs, 2003). Because the in vitro experiments were performed in human embryonic kidney (HEK293) cells singly expressing MOR and DOR, it may be speculated that the in vivo behaviour of the multitarget ligand LP1 could be a consequence of MOR–DOR heteromer targeting (Pasfernak and Don, 2011). The formation of these heteromers is supported by the existence of functional and physical cross-interactions between MOR and DOR (Cahill et al., 2007; Wang et al., 2010; Zhang and Pan, 2010). MOR–DOR heteromers have been detected in the nervous system using specific antibodies (Gupta et al., 2010) and seem to be an antinociceptive unit distinct from MOR and DOR with different binding pocket and signalling properties (van Rijn et al., 2010). It has also been demonstrated that standard opioid agonists selectively activate heteromers in cultured cells with higher efficacy than they activate homomeric opioid receptors (Yekkira et al., 2010).

Conclusions

The results here reported showed that LP1 acts as a multitarget opioid ligand at the supraspinal level. Opioids that combine MOR agonist–DOR antagonist activity (Purington et al., 2011; Balboni et al., 2010) or MOR agonist–DOR agonist activity (Lee et al., 2011; Vandormael et al., 2011) may be effective antinociceptive agents that are able to attenuate MOR-mediated side effects. In this regard, the data collected for LP1 are consistent with its low capability to induce tolerance (Pasquinucci et al., 2012).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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