Pharmacology, Biochemistry and Behavior 94 (2009) 70–74

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Sedation and antinociception induced by a new pyrazolo[3,4-*b*]pyrrolo[3,4-*d*]pyridine derivative (LASSBio-873) is modulated by activation of muscarinic receptors

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ARTICLE INFO

Article history:
Received 2 April 2009
Received in revised form 7 July 2009
Accepted 19 July 2009
Available online 24 July 2009

Keywords:
Sedation
Muscarinic receptor
Pyrazolo[3,4-*b*]pyrrolo[3,4-*d*]pyridine derivatives
Zolpidem
Antinociception

ABSTRACT

New substances designed for the treatment of anxiety have previously been synthesized, which resulted in the identification of four new pyrazolo[3,4-*b*]pyrrolo[3,4-*d*]pyridine derivatives structurally designed by using zolpidem as lead compound. Among them, LASSBio-873 was the most potent to produce analgesic, sedative and hypnotic effects. Thus, we investigated the possible mechanisms involved in LASSBio-873-induced sedation, as well as its effects on different models of inflammatory pain. LASSBio-873 (4 mg/kg) reduced locomotor activity of mice in the open field test from 205.2 ± 25.6 to 87.6 ± 16.2 movements/min. Atropine, a non-selective muscarinic antagonist, prevented the LASSBio-873-induced sedation and increased locomotor activity to 192.9 ± 30.2 movements/min. In the formalin test, LASSBio-873 (4 mg/kg) significantly reduced the duration of nociceptive behavior during the inflammatory phase, reducing the control reactivity from 197.6 ± 14.5 s to 84.4 ± 10.3 s. Carrageenan reduced the latency for the animal reaction from 5.1 ± 0.2 s (control) to 2.1 ± 0.3 s which was completely reverted by LASSBio-873 (6 mg/kg) to 5.6 ± 0.6 s. Atropine prevented the LASSBio-873-induced antinociceptive and antihyperalgesic activities, indicating the interference of the cholinergic system. LASSBio-873 is a novel prototype of drug that modulates muscarinic activity and could be used for neuropsychiatric and cognitive disorders and other conditions associated to acute and chronic pain.

1. Introduction

According to the Pan American Health Organization, mental disorders are affecting 1 in 4 individuals at some time during their lives and the incidence is higher among patients in primary health care. Mental disorders impact adversely on the quality of their lives, families and communities. In the scope of a research aimed to develop new alternatives to treat neurological disorders, new functionalized pyrazolo[3,4-*b*]pyrrolo[3,4-*d*]pyridine derivatives were synthesized by using zolpidem as lead compound (Menegatti et al., 2006). These new compounds were originally designed to be selective GABA receptor modulators exploring its structural analogy with zolpidem, which produces hypnosis of rapid onset and short duration as a consequence of its binding to benzodiazepine receptors (Sanger and Depoortee, 1998). Additionally, zolpidem decreases locomotor activity of adult mice (Depoortere et al., 1986; Fahey et al., 2006; Pericić et al., 2008; Sanger et al., 1986; Tanaka et al., 2008) and induces analgesia at high doses (80 mg/kg) (Pick et al., 2005). Although the mechanisms responsible for the zolpidem-induced analgesia are still unknown, the involvement of opioid pathway has also been suggested (Pick et al., 2005).

The pharmacological profile of the new derivatives (LASSBio-873, LASSBio-872, LASSBio-981, LASSBio-980) was previously evaluated and they produced intense sedative, hypnotic and analgesic activities. Except for LASSBio-980, all derivatives significantly altered the spontaneous locomotor activity in mice and all four new heterotricyclic compounds increased the duration of hypnosis induced by pentobarbital sodium. The antinociceptive effect of the new derivatives was prevented by the treatment with naloxone, indicating that this bioprofile was dependent on the activation of opioid receptors (Menegatti et al., 2006). Among the new derivatives, LASSBio-873 appeared to be the most potent compound to produce sedative and antinociceptive activity, and thus, was selected for additional pharmacological investigation including the determination of the pathways involved in its effects.

The present work investigated the involvement of opioid, GABAergic, cholinergic and adrenergic systems in the sedative activity of LASSBio-

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doi:10.1016/j.pbb.2009.07.008

873. Besides, we evaluated the antinociceptive effects of LASSBio-873 in different models of inflammatory pain and possible mechanisms of action.

2. Materials and methods

The Animal Care and Use Committee at Universidade Federal do Rio de Janeiro approved the protocols used.

2.1. Animals

Male Swiss mice, weighing 20–25 g were housed under an artificial 12-h light and 12-h dark cycle in controlled conditions of temperature (21 °C) and humidity (60%) with food and water ad libitum.

2.2. Drugs

Naloxone and flumazenil were kindly donated by Cristália Produtos Químicos e Farmacêuticos (São Paulo, SP, Brazil). Carrageenan, formaldehyde, indomethacin, acetyl salicylic acid, atropine, yohimbine and pirenzepine were purchased from Sigma Chemical (St Louis, MO, USA). All compounds were dissolved in distilled water, except for flumazenil, indomethacin and the LASSBio-873, which were dissolved in dimethyl sulphoxide (DMSO).

2.3. Methods

2.3.1. Locomotor activity test in mice

The sedative activity of the vehicle and LASSBio-873 was investigated by recording spontaneous locomotor activity of mice in an open field (Menegatti et al., 2006). Spontaneous locomotor activity was determined in Swiss mice (20–25 g) which were placed in the center of an open field of 45×45 cm (LE 8811, Letica) in which 16 infrared photocells were positioned every 2.5 cm. Total locomotor activity was defined as the number of interruptions of the beams registered in a computer during a 40 min period after intraperitoneal injection of either vehicle or LASSBio-873 (4 mg/kg) in a group of ten mice. Data were expressed as the number of movements per minute. To investigate the mechanisms involved in the sedative activity of derivatives, animals were pre-treated intraperitoneally with the following antagonists: naloxone 1 mg/kg, an opioid antagonist (Zomkowski et al., 2005); flumazenil 10 and 20 mg/kg, a $GABA_A$ antagonist (Savić et al., 2004); yohimbine 5 mg/kg, an α_2-adrenergic antagonist (Kaur et al., 2007); atropine 2 mg/kg, a muscarinic antagonist (Adamik and Telegdy, 2004); pirenzepine 10 mg/kg, a selective antagonist of the M_1 muscarinic receptor subtype (Yin and Zhu, 2005).

2.3.2. Formalin test

Formalin test was performed based on protocol developed for Dubuisson and Dennis (1997). Formalin (20 µl, 2.5%) was administered by intraplantar injection into the right hind paw of each animal after 15 min of the intraperitoneal injection of vehicle, acetyl salicylic acid, and LASSBio-873. After that the animal was placed in a plexiglass cage and a mirror was placed under the cage to allow full view of the hind paws and total time spent by animal licking or biting the injected paw was observed for the following 30 min. Formalin-induced pain behavior is biphasic. The initial acute phase (0–5 min) is followed by a short quiescent period; which is then followed by a prolonged tonic response (15–30 min). The involvement of different pathways in the effect of LASSBio-873 was investigated by the pre-treatment with atropine 2 mg/kg, i.p. (Anjaneyulu and Chopra, 2006), naloxone 1 mg/kg (Pini et al., 1997), flumazenil 20 mg/kg (Knabl et al., 2009) or yohimbine 5 mg/kg (Anjaneyulu and Chopra, 2006) 15–30 min before the administration of the derivative.

2.3.3. Carrageenan test

The thermal hyperalgesia was evaluated based on a described method for Hargreaves et al. (1988). The animals were placed in a plexiglass chamber on a glass plate under which a radiant heat stimulus was applied to the hind paws through the glass plate. The light beam was turned off when the animal lifted the paw, allowing the measurement of time between start of the light beam and the paw lift. This time was defined as the paw withdrawal latency, which was assessed using a plantar analgesia meter (ITC Inc. model 33). The stimulus was adjusted to give 5–6 s withdrawal latency in the control condition. For all mice both hind paws were tested three times to obtain average response latency for each hind paw.

Peripheral inflammation was induced by intraplantar (i.pl.) injection of carrageenan (20 µl, 1%) into the right hind paw in mice. The latency of each animal to respond to the thermal stimuli was measured at different time points after carrageenan injection. LASSBio-873, vehicle and indomethacin were administrated i.p. right before carrageenan. A cutoff time of 15 s was used to avoid tissue damage to the hind paw. In order to investigate the mechanism of action of LASSBio-873, animals were pre-treated intraperitoneally with different antagonists, as described for the formalin test above.

2.4. Statistical analysis

Data were presented as mean ± SEM. Comparison of groups was performed using one-way analysis of variance (ANOVA), followed by Dunn's test. Difference was considered statistically significant when $P<0.05$.

3. Results

The sedative properties of LASSBio-873 were investigated by recording spontaneous locomotor activity of mice in an open field. In this test, compounds with sedative activity produce a decrease in the number of movements, interpreted as a decrease in curiosity of the new environment (Prut and Belzung, 2003). The i.p. administration of LASSBio-873 (4 mg/kg) decreased locomotor activity from 205.2 ± 25.6 movements/min (DMSO) to 87.6 ± 16.2 (Fig. 1). In order to investigate the mechanisms involved in the sedation induced by LASSBio-873, mice were pre-treated with different antagonists. The

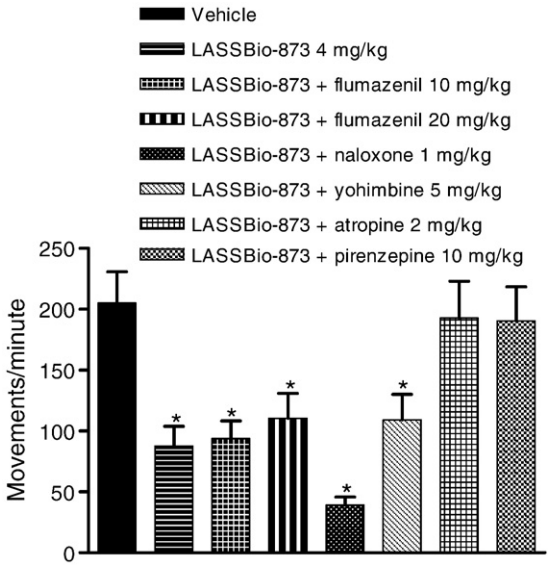

Fig. 1. Effects of LASSBio-873 on the locomotor activity in mice in the absence and presence of antagonists. Derivative was injected intraperitoneally and motor activity was determined during 40 min after injection. Data are expressed as means of the movements per minute ± SEM. *$P<0.05$ relative to control group (DMSO).

involvement of the benzodiazepine pathway was evaluated through pre-treatment with flumazenil (10 and 20 mg/kg, i.p.), 15 min prior to the administration of derivatives. Administration of flumazenil alone significantly reduced the motor activity in mice when compared with vehicle and did not alter the inhibitory effect of derivatives. Even after pre-treatment with a higher dose of flumazenil (20 mg/kg), LASSBio-873 reduced locomotor activity to 93.8 ± 14.4. Pre-treatment with naloxone (1 mg/kg, i.p.), a non-selective opioid antagonist, did not inhibit the derivative-induced sedation but increased its inhibitory effect. Similarly to flumazenil, naloxone alone decreased the locomotor activity of mice.

The participation of the α_2-adrenergic pathway was assessed by the pre-treatment with yohimbine (5 mg/kg, i.p.), which did not antagonize the effect of LASSBio-873. In order to determine the involvement of the muscarinic system in the sedative activity, mice were pre-treated with atropine (2 mg/kg, i.p.). Administration of yohimbine (203.7 ± 18.7 movements/min) and atropine (211.9 ± 30.6 movements/min) alone did not alter locomotor activity in mice. Pre-treatment with atropine significantly antagonized the derivative-induced sedation, as shown in Fig. 1. Locomotor activity was 192.9 ± 30.2 movements/min for LASSBio-873, in the presence of atropine. Similarly, pre-treatment of mice with pirenzepine, a selective antagonist of the M1 muscarinic receptor subtype, completely reversed the sedative effect of LASSBio-873 (190.6 ± 27.9 movements/min), indicating the importance of these receptors for the activity of the derivative.

Intraplantar injection of 20 µl of formalin (2.5%) generated a classical biphasic nociceptive response (Fig. 2). The administration of acetyl salicylic acid (150 mg/kg, i.p.), a non-steroidal antinflammatory agent, and LASSBio-873 (4 mg/kg) 15 min before formalin significantly reduced phase 2 but not phase 1 of the formalin test (Fig. 2). LASSBio-873 reduced reactivity to formalin from 197.6 ± 14.5 s to 84.4 ± 10.3 s ($P<0.05$). Atropine (2 mg/kg, i.p.), a muscarinic receptor antagonist, administered 40 min before formalin, prevented the antinociceptive effect of LASSBio-873 demonstrated in the inflammatory phase (Fig. 2). The effect of LASSBio-873 on the reactivity to formalin was not blocked by 20 mg/kg flumazenil (66.3 ± 12.4 s), 5 mg/kg yohimbine (74.5 ± 17.3 s) and 1 mg/kg naloxone (36.2 ± 12.0 s). Pre-administration of all antagonists tested, except yohimbine, resulted in reactivity that was not significantly different than that produced by formalin alone.

The intraplantar injection of carrageenan induced thermal hyperalgesia in the ipsilateral hind paw, which was consistent with previous studies (Hargreaves et al., 1988; Shannon et al., 2001; Zhang et al., 2004). The i.p. injection of the vehicle alone did not alter the hyperalgesia induced by carrageenan, which reduced baseline withdrawal from 5.1 ± 0.2 to 2.0 ± .0.1 s (after 30 min) (Fig. 3). Indomethacin (2 mg/kg, i.p.) produced total recovery of carrageenan-induced hyperalgesia during 180 min. At 4 mg/kg, the heterotricyclic derivative LASSBio-873 inhibited the development of thermal hyperalgesia induced by carrageenan for 45 min. The latency was recovered to 5.0 ± 0.5 s after 30 min. At 6 mg/kg, the derivative abolished the hyperalgesic effect of carrageenan during 180 min similarly to indomethacin.

To demonstrate if the antinociceptive effect of LASSBio-873 was also related with the activation of muscarinic system, atropine (2 mg/kg, i.p.) was administrated 30 min before the derivative. At 6 mg/kg, the derivative could abolish carrageenan-induced nociceptive effect during all protocol, with a maximal effect after 30 min, when the latency was 5.6 ± 0.6 s. As shown in Fig. 3, in the presence of atropine, latency was 2.2 ± 0.2 s, indicating the involvement of the cholinergic system. Flumazenil (20 mg/kg), yohimbine (5 mg/kg) and naloxone (1 mg/kg) did not modify the effect of LASSBio-873 on carrageenan test. Latency for paw withdrawal was 5.1 ± 0.9, 4.6 ± 0.4 and 4.9 ± 0.5 s, respectively. Administration of all antagonists alone did not interfere with the carrageenan test.

4. Discussion

In the present work we demonstrated that unlike zolpidem, whose sedative and hypnotic profile is related to the benzodiazepine pathway, LASSBio-873-induced sedation may involve the activation of the cholinergic system since it was prevented by the non-selective muscarinic antagonist atropine and by the selective M_1 subtype antagonist pirenzepine.

It is generally believed that locomotor activity results from brain activation, which is manifested as an excitation of central neurons and an increase in cerebral metabolism. While different neurochemical mechanisms are involved in brain activation, dopamine appears to play an essential role in this process (Le and Simon, 1991; Lee et al., 2008; Salamone et al., 2005). Likewise, a proper balance between striatal cholinergic and dopaminergic neurotransmission is required for coordinated locomotor control (Calabresi et al., 2000; Di Chiara et al., 1994; Graybiel, 1990). Acetylcholine-mediated neurotransmission has a crucial role in the control of voluntary movement exerted by the striatum and, accordingly, this brain area contains some of the highest level of acetylcholine and muscarinic receptors in the central nervous system (Calabresi et al., 2000; Graybiel et al., 1994; Hersch

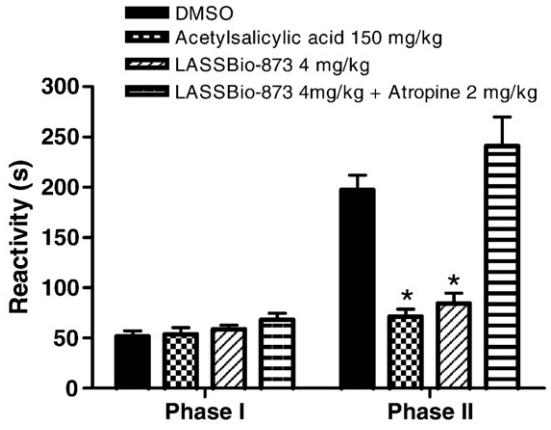

Fig. 2. Effects of LASSBio-873 on the formalin test. Derivative was injected intraperitoneally 15 min before formalin, the antinociceptive activity was observed for 30 min. Data are expressed as means of reactivity (time spent by animal licking or biting the injected paw) ± SEM. *$P<0.05$ relative to control group (DMSO).

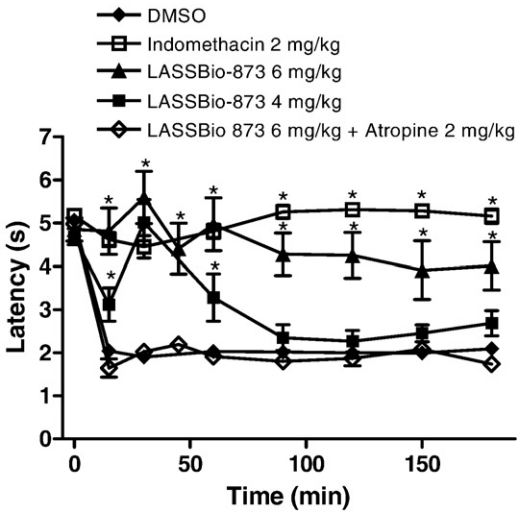

Fig. 3. Effects of LASSBio-873 on the carrageenan test. Derivative was injected intraperitoneally right before carrageenan, the antihyperalgesic activity was observed for 180 min. Data are expressed as means of latency of withdrawal threshold ± SEM. *$P<0.05$ relative to control group (DMSO).

et al., 1994). It is assumed that acetylcholine acting at muscarinic receptors exerts an inhibitory effect on dopaminergic neurotransmission in the striatum (Eckmann et al., 1988). M_1 and M_3 receptors promote phosphatidyl inositol hydrolysis and intracellular Ca^{2+} mobilization, whereas M_2 receptors are negatively coupled to adenylate cyclase activity (Hulme et al., 1990). These receptors exert both presynaptic and postsynaptic effects on striatal cells and mediate opposite effects on striatal synaptic plasticity (Calabresi et al., 2000). Studies have shown that M_1 receptor-deficient mice have significantly elevated levels of extracellular dopamine in the striatum and that this is most likely attributable to M_1 receptors located on extrastriatal neurons projecting to the striatum (Zhang et al., 2002). All these facts indicate that activation of muscarinic receptors is involved in the regulation of locomotor activity.

Besides sedative profile, LASSBio-873 also showed pronounced antinociceptive and antihyperalgesic activities in two different models of inflammatory pain, formalin and carrageenan tests. The subcutaneous injection of formalin produces a biphasic response characterized by an immediate and intense increase in the spontaneous activity followed by a quiescent phase and then a more prolonged increase in cell firing of both primary afferents (Heapy et al., 1987; Puig and Sorkin, 1995; Shannon et al., 2001) as well as dorsal horn neurons (Dickenson and Sullivan, 1987). LASSBio-873 only significantly reduced the late phase of formalin test and this effect was reverted by the pre-treatment with atropine. Similarly, the cholinesterase inhibitor physostigmine was shown to reduce the second phase of formalin-induced pain, which was prevented by atropine pre-treatment (Mojtahedin et al., 2008). Moreover, studies have shown that non-selective muscarinic agonists reduce the responses to formalin (Capone et al., 1999; Barocelli et al., 2001; Shannon et al., 2001).

Carrageenan produces an inflammation that can generate a state of spinal cord sensitization in which noxious stimulus produces an exaggerated response (hyperalgesia) and a normally non-noxious stimulus can produce a nocifensive response (allodynia) to both thermal and mechanical stimuli (Hargreaves et al., 1988). This central sensitization is accompanied by increases in the magnitude of C-fiber evoked responses and decreases in descending inhibition without increases in spontaneous firing of C-fiber afferents (Stanfa et al., 1992; Traub, 1997). The effect of LASSBio-873 on carrageenan test was also prevented by atropine, indicating that, as in the locomotor activity test, LASSBio-873-induced antinociception seems to involve the activation of the cholinergic system.

Modulation of antinociception can occur through different physiological systems, including opioid, adrenergic, cholinergic and GABAergic systems. Moreover, interaction and crosstalk between pathways is important for pain signalling. However, the antinociceptive effects of LASSBio-873 seem to be mediated primarily by the cholinergic system, since they were only blocked by atropine.

Muscarinic cholinergic receptors are abundant throughout pain pathways from dorsal root ganglia to somatosensory cortex and therefore could modulate processing of sensory information at several levels (Shannon et al., 1997; Tata et al., 2000). Muscarinic receptors are specially localized on the superficial laminae of the dorsal horn in rats where they terminate nociceptive fibers Aδ and C (Abelson and Höglund, 2002; Shannon et al., 2001). By mimicking the release of ACh in the spinal cord, muscarinic agonists can reduce the release of glutamate (Li et al., 2002; Jones and Dunlop, 2007; Pan et al., 2008) attenuating the excitatory signals, and can enhance the inhibitory GABAergic tone (Li et al., 2002, Pan et al., 2008). In addition to the descending inhibition through direct activation of the dorsal horn neurons, muscarinic agonists may also act indirectly by modulating the nitric oxide pathways (Eisenach, 1999).

The precise mechanisms through which LASSBio-873 exerts its action still remain unclear, but activation of the cholinergic system seems largely to account for its sedative and antinociceptive activities (formalin and carrageenan tests). The cholinergic system offers a number of possible targets for pain transmission and nervous system activity modulation, indicating its therapeutic potential for the development of new drugs. LASSBio-873 is a novel prototype of drug that modulates muscarinic activity and could be used for neuropsychiatric and cognitive disorders and other conditions associated to acute and chronic pain.

Acknowledgements

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, BR), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, BR), Fundação Universitária Jose Bonifácio (FUJB, BR), Pensa Rio - Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, BR) and Instituto Nacional de Ciência e Tecnologia/Fármacos (CNPq/FAPERJ, BR) for finantial support and fellowships from CAPES (to JMR, TFM) and CNPq (to GZS, RTS, NMNJ, CAMF, EJB).

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