Impairment of locomotor activity induced by the novel N-acylhydrazone derivatives LASSBio-785 and LASSBio-786 in mice

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Abstract

The N-acylhydrazone (NAH) analogues N-methyl 2-thienylidene 3,4-benzoylhydrazine (LASSBio-785) and N-benzyl 2-thienylidene 3,4-benzoylhydrazine (LASSBio-786) were prepared from 2-thienylidene 3,4-methylenedioxybenzoylhydrazine (LASSBio-294). The ability of LASSBio-785 and LASSBio-786 to decrease central nervous system activity was investigated in male Swiss mice. LASSBio-785 or LASSBio-786 (30 mg/kg, ip) reduced locomotor activity from 209 ± 26 (control) to 140 ± 18 (P < 0.05) or 146 ± 15 crossings/min (P < 0.05), respectively. LASSBio-785 (15 or 30 mg/kg, iv) also reduced locomotor activity from 200 ± 15 to 116 ± 29 (P < 0.05) or 60 ± 16 crossings/min (P < 0.01), respectively. Likewise, LASSBio-786 (15 or 30 mg/kg, iv) reduced locomotor activity from 200 ± 15 to 127 ± 10 (P < 0.01) or 96 ± 14 crossings/min (P < 0.01), respectively. Pretreatment with flumazenil (20 mg/kg, ip) prevented the locomotor impairment induced by NAH analogues (15 mg/kg, iv), providing evidence that the benzodiazepine (BDZ) receptor is involved. This finding was supported by the structural similarity of NAH analogues to midazolam. However, LASSBio-785 showed weak binding to the BDZ receptor. LASSBio-785 or LASSBio-786 (30 mg/kg, ip, n = 10) increased pentobarbital-induced sleeping time from 42 ± 5 (DMSO) to 66 ± 6 (P < 0.05) or 75 ± 4 min (P < 0.05), respectively. The dose required to achieve 50% hypnosis (HD50) following iv injection of LASSBio-785 or LASSBio-786 was 15.8 or 9.5 mg/kg, respectively. These data suggest that both NAH analogues might be useful for the development of new neuroactive drugs for the treatment of insomnia or for use in conjunction with general anesthesia.

Key words: N-acylhydrazone; Locomotor activity; Sedation; Hypnosis; Benzodiazepine receptor; Flumazenil

Introduction

During an ongoing research program aimed at developing novel treatments for cardiovascular disorders, novel N-acylhydrazone (NAH) compounds based on 2-thienylidene 3,4-methylenedioxybenzoylhydrazine (LASSBio-294) were synthesized. LASSBio-294 was synthesized from safrole (1,2), an abundant Brazilian natural product obtained from Ocotea pretiosa (3). LASSBio-294 was prepared as a bioactive 6-aryl-4,5-heterocyclic-fused pyridazinone compound, part of a family of compounds known as potent and selective phosphodiesterase inhibitors (4), and its cardiac inotropic properties were evaluated. LASSBio-294 exhibited significant positive cardiac inotropic activity due to increased calcium accumulation in the sarcoplasmic reticulum (5).

Additionally, LASSBio-294 induced the relaxation of aortic rings, an effect mediated by the guanylate cyclase/cyclic guanylate monophosphate pathway (6).

In order to identify new drug candidates with enhanced vasodilatory properties and decreased side effects, two novel analogues of LASSBio-294 were synthesized by the introduction of a methyl and a benzyl group in the amide nitrogen of the NAH moiety, to yield LASSBio-785 (N-methyl 2-thienylidene 3,4-benzoylhydrazine) and LASSBio-786 (N-benzyl 2-thienylidene 3,4-benzoylhydrazine), respectively. LASSBio-785 exhibited improved vasodilator properties (IC50: 10.2 ± 0.5 μM) and was seven times more potent than LASSBio-294 (74 μM) in aortic rings pre-contracted with phenylephrine (7), and...
both derivatives were recently shown to inhibit platelet aggregation (8). While investigating the hemodynamic effect of LASSBio-785 in rats, we noted that the animals fell asleep, an effect not observed with the LASSBio-294 prototype compound. Therefore, the current study was designed to investigate the sedative-hypnotic pharmacological properties of LASSBio-785 and the more lipid-soluble LASSBio-786. We also investigated the involvement of the GABAergic system in the sedative-hypnotic activities of both compounds.

Material and Methods

Animals

Male Swiss mice (20-25 g) were housed in a temperature- (24 ± 1°C) and humidity-controlled (60%) room with a 12-h light/dark cycle. The mice were fed a standard diet with water ad libitum. Housing, handling and experimental procedures were in compliance with the recommendations of the Animal Care and Use Committee of Universidade Federal do Rio de Janeiro (protocol #DFBCICB013). The animals were randomly assigned to a single-treatment group and were used only once.

Drugs

Midazolam, sodium pentobarbital and flumazenil were generously donated by Cristália Produtos Químicos e Farmacêuticos (Brazil). LASSBio-785 and LASSBio-786 were generously donated by Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio, Universidade Federal do Rio de Janeiro, Brazil). All compounds were dissolved in dimethyl sulfoxide (DMSO), except for midazolam, which was dissolved in distilled water, and pentobarbital sodium, which was dissolved in propylene glycol and distilled water.

Methods

Assessment of locomotor activity in mice. Spontaneous locomotor activity was measured in an open field (45 × 45 cm, LE 8811, Panlab, USA) in which 16 infrared photocells were positioned every 2.5 cm. The mice were placed individually in the center of the chamber. The sedative properties of midazolam, LASSBio-785 and LASSBio-786 were compared using locomotor activity as an index of sedation (9). Locomotor activity was quantified as the number of beam interruptions (crossings) registered by a computer, and data are reported as the number of crossings per minute (crossings/min). After ip or iv injection of vehicle (DMSO), midazolam (2 mg/kg), LASSBio-785 (15 or 30 mg/kg), or LASSBio-786 (15 or 30 mg/kg), locomotor activity was recorded cumulatively over a 40-min session, divided into 8 5-min periods. The doses of derivatives were selected on the basis of preliminary tests with LASSBio-785 (data not shown). Each treatment group consisted of 10 mice. The same positive (midazolam) and negative (vehicle) control groups were used to analyze the effects of LASSBio-785 and LASSBio-786 on locomotor activity.

In another set of experiments, in order to investigate the mechanism involved in the sedative activity of LASSBio-785 and LASSBio-786, the mice were pretreated with a specific antagonist of the benzodiazepine receptor on the GABA_A receptor complex (flumazenil, 20 mg/kg, ip) 15 min before the administration of LASSBio-785 or LASSBio-786 (15 mg/kg, iv) (10).

Assessment of pentobarbital-induced sleeping time in mice. Mice were gently positioned in a restraining tube, and sodium pentobarbital (30 mg/kg) was injected intravenously into the tail vein. The time from the loss of the up-righting reflex to its recovery was recorded as the sleeping time. Three consecutive trials up to recovery of this reflex were used to determine the hypnosis endpoint (11). To investigate the hypnotic activity, LASSBio-785 (15 or 30 mg/kg, ip) or LASSBio-786 (15 or 30 mg/kg, ip) was injected 15 min before sodium pentobarbital and the sleeping time was determined as before. Injection of midazolam (2 mg/kg, ip) 15 min before sodium pentobarbital (30 mg/kg, iv) was used as a positive control. Another control group received DMSO (ip) 15 min before sodium pentobarbital administration.

Assessment of LASSBio-785 and LASSBio-786 hypnotic activity in mice. Mice were injected intravenously with LASSBio-785 (10, 15, 20, and 25 mg/kg) or LASSBio-786 (5, 10, 15, and 20 mg/kg) in order to determine the HD_{50} for the hypnotic activity of the compounds based on loss of the righting reflex. Groups of 10 animals were used for each dose. Solutions were prepared immediately before the test. Logarithmic dose-response curves for LASSBio-785 and LASSBio-786 were fitted to calculate the HD_{50} using a linear regression analysis.

Root mean square (RMS) analysis. The structural similarities of LASSBio-785 and LASSBio-786 to the benzodiazepine (BDZ) drug midazolam were first estimated using the BioMedCAChé 6.0 software. The most stable synperiplanar structural conformers (12) were then superimposed on the corresponding BDZ scaffolds, thus aligning the central NAH framework. The RMS of midazolam was then calculated to enable evaluation of the structure-activity relationships between BDZ and the novel analogues, LASSBio-785 and LASSBio-786. The closer to zero the RMS values are, the higher the structural correlation between them.

Binding assay

A binding assay was performed between LASSBio-785 and the BDZ receptor site (13) or the GABA-gated Cl-channel of the GABA_A receptor complex (14) of the rat cerebral cortex using a single concentration (50 μM) [3H]-flunitrazepam (0.4 nM) and [35S]-TBPS (3 nM) were used as the BDZ agonist radioligand and the Cl-channel.
GABA-gated antagonist radioligand, respectively. Data are reported as percent inhibition of specific binding afforded by LASSBio-785 relative to control, using the equation: % inhibition = 100 - [(measured specific binding / control specific binding) x 100].

Statistical analysis
Data are reported as means ± SE. The Student t-test was used to compare the differences between the positive control and vehicle groups at the same time. Comparisons between vehicle (control) and treatment groups were analyzed by one-way analysis of variance (ANOVA), followed by the Dunnett multiple comparison test. Differences with P < 0.05 were considered to be statistically significant. Statistical analyses were performed using the GraphPad Prism® 4.0 software (USA).

Results
Effects of LASSBio-785 and LASSBio-786 on the locomotor activity of mice
The potential sedative activity of LASSBio-785 and LASSBio-786 at doses of 15 and 30 mg/kg (ip and iv) was evaluated based on measurements of spontaneous locomotor activity of mice in an open field. Figure 1A shows that ip administration of LASSBio-785 at 15 mg/kg did not significantly reduce the locomotor activity relative to DMSO-treated control mice. However, at a higher dose of LASSBio-785 (30 mg/kg), locomotor activity was reduced 30%. Midazolam (2 mg/kg, ip), which was used as a BDZ-like positive control, significantly reduced locomotor activity 45%. Intraperitoneal administration of LASSBio-786 at 15 and 30 mg/kg also reduced locomotor activity 29 and 30%, respectively (Figure 1B).

When injected intravenously at 15 and 30 mg/kg, LASSBio-785 decreased locomotor activity from 200 ± 15 (DMSO, n = 10) to 116 ± 29 (P < 0.05, n = 10) and 60 ± 16 crossings/min (P < 0.01, n = 10), respectively. Midazolam treatment (2 mg/kg, iv) reduced locomotor activity to 120 ± 19 crossings/min (P < 0.01, n = 10). The same effect was observed after iv administration of 15 and 30 mg/kg LASSBio-786, when locomotor activity decreased to 127 ± 10 (P < 0.01, n = 10) and 96 ± 14 crossings/min (P < 0.01, n = 10), respectively.

The involvement of the BDZ pathway was investigated by examining the influence of pretreatment with flumazenil (20 mg/kg, ip) on the sedative properties of LASSBio-785 (15 mg/kg, iv) and LASSBio-786 (15 mg/kg, iv). Flumazenil did not significantly reduce locomotor activity when compared to the vehicle-pretreated group (170 ± 19 and 209 ± 26 crossings/min, respectively, n = 10). Locomotor activity following LASSBio-785 administration to vehicle- or flumazenil-pretreated mice was 116 ± 29 and 186 ± 5 crossings/min (P < 0.05, n = 10), respectively. Likewise, flumazenil pretreatment also attenuated the depression of locomotor activity induced by LASSBio-786 (from 127 ± 10 to 174 ± 9 crossings/min, P < 0.05, n = 10).

Effects on pentobarbital-induced sleeping time in mice
Intravenous injection of sodium pentobarbital (30 mg/kg) to DMSO-treated (ip) control mice induced sleeping time of 42 ± 5 min (Figure 2). Sleeping time was increased in mice pretreated with midazolam (2 mg/kg, ip). Administration of LASSBio-785 and LASSBio-786 at 15 mg/kg (ip) did not alter sodium pentobarbital-induced sleeping time significantly, but at 30 mg/kg these compounds prolonged sleeping time (Figure 2).

Effects on the hypnotic activity in mice
Intravenous injections of 10, 15, 20, and 25 mg/kg LASSBio-785 induced loss of the righting reflex 20, 40,
70, and 90% of each group, respectively. In mice similarly treated with LASSBio-786 at 5, 10, 15, and 20 mg/kg, the reflex loss was 20, 60, 70, and 80%, respectively. The HD50 values for LASSBio-785 and LASSBio-786, calculated from the log dose-response curves, were 15.8 and 9.5 mg/kg, respectively (Figure 3).

**Superimposition of NAH derivatives and BDZ compounds**

Figure 4 shows the occurrence of significant structural correlations in pharmacologically relevant regions between the N-alkyl NAH derivatives and the BDZ drug midazolam. The RMS values calculated after superimposing the structures of LASSBio-785 and LASSBio-786 on that of midazolam were 0.358 and 0.378, respectively.

**Binding assay**

In rat cerebral cortex membranes, LASSBio-785 (50 µM) inhibited binding of the antagonist [3H]-flunitrazepam (0.4 nM) to BDZ receptors by 14.2 and 8% (average 11.1%, n = 2) and of the antagonist [35S]-TBPS (3 nM) to Cl-channel GABA_A-gated receptors by 11.2 and 17.6% (average 14.4%, n = 2). LASSBio-786 was not tested in these assays.

**Discussion**

The main finding of the current study was that LASSBio-785 and LASSBio-786, which respectively have a methyl or benzyl group linked to the amide nitrogen unit of the NAH moiety of LASSBio-294, a cardiac ionotropic (5) and vasodilator compound (6), significantly reduced the locomotor activity of mice measured in an open-field, a protocol that has been used to efficiently monitor the sedative effect of drugs (15). In addition, the effects of both derivatives were sensitive to inhibition by pretreatment with the BDZ receptor antagonist flumazenil and were accompanied by prolongation of the hypnotic effects of pentobarbital-induced sleeping time, but no anxiolytic-like effects were detected with either derivative in the elevated plus-maze test. Inhibition of neuronal excitability by the neurotransmitter GABA is mainly the result of activation of GABA_A receptors, pentameric ligand-gated chloride channels which, via enhanced chloride influx, induce hyperpolarization and reduce firing of action potentials (16). These inhibitory effects of GABA are facilitated by BDZ agonists, which act allosterically on the GABA_A receptor complex to enhance affinity of the GABA binding site to the
neurotransmitter, and it is estimated that 20-30% of the inhibitory neurons in the CNS are GABAergic. There is a wide diversity of GABA\(_A\) receptor subtypes, and those most highly expressed in the CNS include the \(\alpha1\beta2\gamma2\), \(\alpha2\beta2\gamma2\), \(\alpha3\beta2\gamma2\), and \(\alpha5\beta2\gamma2\) isoforms. Previous studies have reported that \(\alpha1\)-containing GABA\(_A\) receptors mediate the sedative, addictive, anterograde amnesic, and part of the anticonvulsant activities of the BDZ diazepam, whereas the \(\alpha2\)-containing isoform is the main mediator of its anxiolytic-like effects and also promotes part of its myorelaxant actions, alongside the \(\alpha3\)- and \(\alpha5\)-containing isoforms (17). The \(\alpha5\)-containing GABA\(_A\) receptors have also been linked to the development of tolerance to the sedative effects of BDZ (17).

LASSBio-785 and LASSBio-786 could facilitate GABAergic inhibition via interactions with the GABA\(_A\) receptor complex. This hypothesis is supported by the finding that the sedative effects of both compounds were reversed by the BDZ receptor antagonist flumazenil. This view is strengthened by the evidence for structural correlation observed by \textit{in silico} superimposition of the pharmacophoric points of LASSBio-785 and LASSBio-786 with the BDZ drug midazolam, as detected by the determination of RMS. Despite these findings, preliminary binding analyses using a single concentration of LASSBio-785 have shown only weak interactions between this compound and the BDZ-binding site. Even if a more detailed investigation regarding characterization of the LASSBio-785 and LASSBio-786 binding sites on the GABA\(_A\) receptor complex is still warranted, it is important to highlight that some currently available sedatives, which are active at \(\alpha1\)-containing GABA\(_A\) receptors, such as zolpidem and zopiclone, do not share structural similarity with BDZs (18).

The insertion of methyl and benzyl groups in the NAH subunit of LASSBio-785 and LASSBio-786 is associated with the synperiplanar conformation of both compounds, whereby the amide hydrogen is synperiplanar to the carbonyl oxygen (12). The present study shows that this folded structure can play an important role in the central depressor activity of these substances.

In contrast to barbiturates, BDZ-like compounds do not directly open the chloride channel (17). Preliminary binding assays with LASSBio-785 (50 \(\mu\)M) have shown

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**Figure 4.** Structural correlation between the N-acylhydrazone (NAH) derivatives LASSBio-785 and LASSBio-786 and the benzodiazepine compound midazolam. The atoms were colored as follows: carbon (gray), hydrogen (white), oxygen (red), and nitrogen (blue). The circles indicate the atoms used for the alignment of the structures. A, Chemical structures of LASSBio-785 (1), LASSBio-786 (2), and midazolam (3). B, Superimposition of compounds 1 vs 3 (yellow structure) and 2 vs 3 (yellow structure), aligned by the central NAH framework.
moderate displacement (average 14.4%) of the antagonist \(^{[35S]}\)TBPS from its specific binding sites on the chloride channel of GABA\(\text{A}\) receptors, suggesting that it may act as an agonist at this target. Thus, the effects of LASSBio-785 (and possibly LASSBio-786) on GABA receptors of the CNS may be associated with both an indirect activation via binding to the BDZ receptor and a direct stimulatory effect on the chloride channel. These mechanisms could explain the increased duration of hypnosis induced by pentobarbital following LASSBio-785 or LASSBio-786 administration.

On the other hand, LASSBio-785 and LASSBio-786 were more potent in reducing locomotor activity when given \(i.v\) as compared to the \(i.p\) route. The less intense sedative effects seen following \(i.p\) administration could be due to poor absorption into the blood stream or to significant liver metabolism. These aspects, as well as the bioavailability of both derivatives following oral administration, remain to be assessed. Moreover, even if LASSBio-786 was deliberately planned to display greater lipid solubility than LASSBio-785 (lipid solubility ClogP values of 3.85 ± 0.62 and 2.09 ± 0.61, respectively) to favor its transfer across the blood-brain barrier, both compounds were equipotent in promoting sedation following \(i.v\) injection.

In addition to their sedative effects, classical BDZ-like compounds can also promote anticonvulsant and anxiolytic-like effects. The possibility that LASSBio-785 and LASSBio-786 could display similar properties was also tested, but the results were negative. At 5 mg/kg, \(i.v\), neither derivative protected mice from seizures induced by pentylenetetrazole (10 or 15 mg/kg, \(i.p\)) or promoted anxiolytic-like behaviors in mice submitted to the elevated plus-maze test (data not shown).

Drug-induced impairment of locomotor activity may be affected by several physiological conditions, including hypotension, pain and muscle relaxation. Although blood pressure was not measured in the current study, rats given \(i.v\) LASSBio-785 infusion at 7.5 mg kg\(^{-1}\)min\(^{-1}\) for 10 min displayed decreases of approximately 30% in diastolic (but not systolic or mean) blood pressure and heart rate over the last 3 min, whereas no such changes were detected following similar infusion of LASSBio-786 (19). Such findings suggest that the locomotor impairment induced by LASSBio-785 and LASSBio-786 was not a consequence of hypotension. Furthermore, since LASSBio-785 and LASSBio-786 can each reduce carrageenan-induced hind paw thermal hyperalgesia (data not shown), it appears unlikely that inhibition of locomotor activity is due to nociceptive effects of these derivatives. In addition, we also observed that the sedative effects of LASSBio-785 and LASSBio-786 were not affected by \(i.p\) pretreatment with either the \(\alpha\)-2-adrenergic antagonist yohimbine (5 mg/kg) or the opioid receptor antagonist naloxone (1 mg/kg) (data not shown). Finally, as LASSBio-785 administration (30 mg/kg, \(i.p\)) did not modify the performance of mice in the rotarod test (data not shown), the inhibition of locomotor activity afforded by this dose of the derivative is unrelated to effects on motor coordination \(per se\).

The therapeutic use of currently available hypnotic drugs is limited by the onset of side effects including risk of addiction and hemodynamic and respiratory depression (17,20). Even if the available reports on the effects of LASSBio-785 and LASSBio-786 show limited (if any) effects on blood pressure, muscle relaxation or impairment of muscular coordination, more studies are needed to compare the toxicological profiles of these compounds with those of hypnotics currently in use in clinical practice.

It is interesting to note that the HD\(_{50}\) values for the hypnotic effects of LASSBio-785 and LASSBio-786 were similar, yet the former derivative is 10-fold less potent than the latter in relaxing isolated arterial vessel rings (7). This difference in the relative potencies of LASSBio-786 and LASSBio-785 in promoting hypnosis and vasodilatation suggests that both effects are the result of actions on distinct targets, and that the CNS effects reported herein are unrelated to modulation of the NO-GMPc system.

Another possible use of LASSBio-785 and LASSBio-786 is as adjuvant drugs in anesthesia. Midazolam and dexmedetomidine have been used successfully to potentiate intravenous and inhalation general anesthetics. The associated increase in pentobarbital-induced sleeping time and the hypnotic effect induced by single-bolus administration indicate that LASSBio-785 and LASSBio-786 may be useful for this purpose.

Two new N-acylhydrazone derivatives of the cardio-inotropic prototype drug LASSBio-294, named LASSBio-785 and LASSBio-786, exhibited sedative and hypnotic properties in mice, which could be potentially relevant towards the development of new neuroactive drugs for the treatment of insomnia and in conjunction with general anesthesia.

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