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Pharmacological Characterization of LB50016, N-(4-Amino)Butyl 3-Phenylpyrrolidine Derivative, as a New 5-HT_{1A} Receptor Agonist

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LB50016 was characterized as a selective and potent 5-HT_{1A} receptor agonist and evaluate its anxiolytic and antidepressant activities. It shows high affinity for 5-HT_{1A} receptor, moderate affinity for α_2 adrenergic and 5-HT_{2A} receptors and no significant affinity for other receptors tested. Hypothermia and increased serum corticosterone level were observed in LB50016-treated rats, which are mediated mostly by post synaptic 5-HT_{1A} receptor activation. In the mouse forced swim model for depression, LB50016-elicited dose-dependent reductions in immobility time, showing ED_{50} of approximately 3 mg/kg i.p., which was blocked by pretreatment of NAN-190, 5-HT_{1A} antagonist. In face-to-face test for anxiolytic activity in mice, estimated ED_{50} was 2 mg/kg, i.p.. In isolation-induced aggression test with mice, fifty-fold increases in latency to attack were observed at 30 min and last up to 4 h after LB50016 treatment (3 mg/kg, i.p.). Taken together, LB50016-induced pharmacological activities are mediated by activation of 5-HT_{1A} receptors, offering an effective therapeutic candidate in the management of anxiety and depression in humans.

Key words : 5-HT_{1A} receptors, Anxiolytic, Antidepressant, LB50016, Pyrrolidine

INTRODUCTION

The 5-HT receptor was proven to be remarkably variable and molecular cloning techniques have allowed characterization of at least seven different types numbered from 5-HT_{1A} to 5-HT_7. Some of them are further divided into subtypes according to their anatomical location or function (Dubovsky and Thomas, 1995; Hoyer and Martin, 1997). Although the classification and localization of 5-HT receptors in neuronal tissues is far from settled, several serotonergic receptors are under investigation as targets for drugs for the treatment of affective disorders, where the 5-HT_{1A} receptor subtype has been figured most prominently in research into depression and anxiety. The 5-HT_{1A} receptors in relation to anxiety and depression have been reviewed by Deakin (Deakin, 1993). In the central nervous system, 5-HT_{1A} receptors are located in pre- and post-synaptic manners (Sharp *et al.*, 1990; Glennon and Dukat, 1991). A variety of compound which have selective 5-HT_{1A} receptor stimulating activity have been identified to be potential antidepressants. These drugs can be divided into several structural groups, the 8-substituted-2-di-n-propylaminotetralins (Schaus *et al.*, 1990; Yu *et al.*, 1993), the aryl-piperazine derivatives (Middlemiss and Tricklebank, 1992; Millan *et al.*, 1992) and the 6-substituted-4-(di-n-propylamino)-1,3,4,5-tetrahydrobenz[c,d]indoles (Flaugh *et al.*, 1988). Expecially, the azapirones are class of drugs being marketed, or in different stages of clinical examination for their efficacy in the treatment of anxiety and depression (Napliello and Domantay 1991; Cadieux, 1996) although there is not yet overall consensus that 5-HT_{1A} agonist are really effective in major depression. The initial success of buspirone, the 5-HT_{1A} receptor agonist in the treatment of generalized anxiety and depression disorders lead us to discover new 5-HT_{1A} agonists with greater selectivity and potency. Our study demonstrates the pharmacological characteristics of LB50016 (Fig. 1), a newly synthesized N-(4-amino)butyl 3-phenylpyrrolidine derivatives as a 5-HT_{1A} agonist assessed with the ligand binding and animal model studies in terms of receptor

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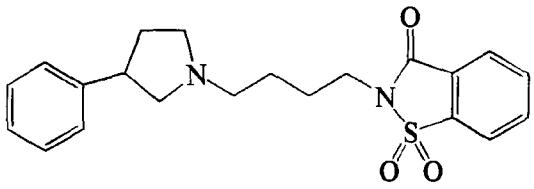

Fig. 1. Structure of LB50016.

selectivity and potential anxiolytic and antidepressant activities.

MATERIALS AND METHODS

Test articles and animals

In order to prepare test article for administration, LB50016 was dissolved in 0.9% saline with brief sonication at 5 mg/ml concentration. Commercially available buspirone was utilized as a reference drug in some experiments. Male Sprague-Dawley rats (250~300 g) and male ICR mice (20~25 g) produced by LG Chem Biotech Institute animal facility (Daejeon, Korea) were used for the following experiments. All animals were housed and fed, *ad libitum*, with a standard commercial food at the LG Chem animal facility fo which environment was well controlled (temperature; 20~22°C; humidity of 45~65%; 12 h light and 12 h dark). All the animals utilized for the oral application studies were fasted overnight prior to treatment.

Drugs

[^{3}H]8-OH-DPAT, [^{3}H]Ketanserin, [^{3}H]RX821002, [^{3}H]SCH23390, [^{3}H]Raclopride, [^{3}H]Pirenzepine and [^{3}H]AFDX-384 (Amersham, Buckinghamshire, England); serotonin, ketanserin, RX821002, butaclamol, buspirone and atropine (RBI Research Biochemicals International, Natick, MA, USA); phenylmethylsulfonylfluoride, ascorbic acid, ethylenediaminetetrascetic acid, leupeptin, aprotinin and all the necessary salts (Sigma Chemicals Co., St. Louis, MO, USA).

Preparation of receptor-rich fractions

Male Sprague-Dawley rats 200~250 g were sacrificed and specific brain regions or heart were used as the sources of binding sites; hippocampus for 5-HT_{1A} receptor, cerebral cortex for 5-HT_{2A}, adrenergic α_2 and muscarinic M_1 receptors, striatum for dopamine D_1 and D_2 receptors, heart for muscarinic M_2 receptor. The tissues were homogenized in 20 volumes of homogenization buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 5 mg/ml pepstatin, 1 mM phenylmethylsulfonylfluoride) using a Polytron at a setting of 17s, 17,000 rpm and sit on ice for 20s. After repeating three times, the homogenates were centrifuged at 18,000 rpm for 15 min in Beckman centrifuge. The resulting pellets were suspended in 20 volumes of homogenization buffer and sit for 10 min at 37°C, followed by centrifugation at 18,000 rpm for 15 min. The final pellets were suspended again in 20 volumes of reaction buffers as described in ligand binding assay, aliquot and frozen at -70°C until the day of assay.

Ligand binding assay

Ligand binding assay was performed to measure binding affinities of LB50016 for the following receptors; 5-HT_{1A} and 5-HT_{2A} receptors, adrenergic α_2 receptor, muscarinic M_1 and M_2 receptors, dopaminergic D_1 and D_2 receptors. The reagents and conditions used in each assays are described in Table I and the methods employed in these studies were described previously (Leysen *et al.*, 1982; Billard *et al.*, 1984; Peroutka, 1986; Hall *et al.*, 1988; Potter *et al.*, 1988; Entzeroth *et al.*, 1990; Orourke *et al.*, 1994). In brief, 200 µl of binding mixture composed of 2 mg of receptor-rich fractions, receptor-specific radioactive ligand, and reaction buffer were incubated for 30 min at 37°C in a 96 well-microplate. Reactions were

Table I. Assay conditions for [^{3}H]ligand binding studies

Receptor	[^{3}H]Ligand [nM]	Nonspecific Ligand [µM]	Receptor Sources	Buffer
5-HT_{1A}	8-OH-DPAT [1]	Serotonin [10]	Hippocampus	A
5-HT_{2A}	Ketanserin [1.5]	Ketanserin [10]	Cerebral Cortex	A
Adrenergic α_2	RX821002 [0.5]	RX821002 [10]	Cerebral Cortex	C
Dopaminergic D_1	SCH23390 [0.5]	Butaclamol [10]	Striatum	B
Dopaminergic D_2	Raclopride [2]	Butaclamol [10]	Striatum	B
Muscarinic M_1	Pirenzepine [2]	Atropine [1]	Cerebral Cortex	C
Muscarinic M_2	AFDX-394 [2]	Atropine [1]	Heart	C

Buffer A; 50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM $CaCl_2$, 0.1% ascorbic acid, 1 mM ethylenediamine tetraacetic acid, 10 µg/ml leupeptin, 5 µm/ml aprotinin.
Buffer B; 50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid, 10 µg/ml leupeptin, 5 µg/ml aprotinin.
Buffer C; 20 mM HEPES, pH 7.4, 110 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 10 µg/ml leupeptin, 5 µg/ml aprotinin.

terminated by vacuum filtration using 96-well cell harvester, followed by washing three times with chilled reaction buffer and filter-bound radioactivity was quantified by liquid scintillation counting. Specific binding was measured by subtracting nonspecific binding out of total binding. Nonspecific binding was measured in the presence of specified concentrations of each non-labeled ligands under the same condition as that for measuring total binding. IC_{50} values were calculated by using nonlinear regresssion analysis and the affinity constants for receptors (Ki values) were calculated from IC_{50} values based on the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Determination of serum corticosterone concentrations

At 60 min after application of LB50016 (3 mg/kg, i. p., 10 mg/kg, p.o., respectively) to rats, animals were decapitated, trunk blood collected, allowed to clot and followed by isolation of serum through spinning at 13,000 rpm, 4°C for 5 min. Obtained serum were stored frozen until anslysis. Serum corticosterone concentrations were determined by utilizing Amersham Biotrak radioimmunoassay kit (Amersham, Buckinghamshire, England). Statistical analysis was performed according to the student t-test, $p<0.05$ as the minimal level of significance.

Effects on body temperature in rats

After LB50016 treatment (3 mg/kg, s.c.) to rats, changes in body temperature were measured for 10~15 s at zero min, 30 min and 60 min with rectal thermometer (Yellow Spring Instrument, USA).

Effects on the forced swim test for antidepressant activity in mice

Experiments were performed as described by Porsolt *et al.* (Porsolt *et al.*, 1977). Briefly, mice stabilized for two weeks in advance in a temperature and humidity controlled conditions were utilized. One day bofore test, each mice was exposed to 5 min swim session for acclimation in transparent glass cylinders (10 cm inner diameter×25 cm in height) containing 6 cm of water (25°C±1°C). At 10 minutes before the forced swim session, various doses of test drugs were given by intraperitoneal injection (1, 3 and 5 mg/kg) and effects were determined by alterations in the immobility time measured for 2 min starting from 2 min after forced swimming in the same cylinder.

Effects on the face-to-face test for anxiolytic activity in mice

The face-to-face test was conducted to evaluate anxiolytic effects of test drugs as described by Schreur *et al.* (Schreur, 1988; McCall *et al.*, 1994). Two mice being kept in different home cages and thus been unfamiliar each other were injected intraperitoneally with the several doses of LB50016 and buspirone. Thirty min later, they were placed together for the first time in a small plastic cage and the face-to-face interaction was observed. Cumulative duration of touch or nearly touch face between each other was measured for 3 min as an index of anxiolytic effects. Drug-induced increases above the cumulative duration of interaction seen for vehicle-treated daily controls is interpreted as an anxiolytic effect.

Effects on the isolation-induced aggression test in mice

Experiments were carried out as described by McCall *et al.* (McCall *et al.*, 1994). Male ICR mice (20~25 g) were housed singly in 12 cm×25 cm cage for several weeks of isolation and aggression training by prodding animals with a forceps, until the animal responded aggressively. The same species of intruder mouse which has been housed in groups of 12 per cage was introduced to the isolated mouse and then the latency to attack by isolated mouse on the intruder was recorded. In order to evaluate an efficacy of LB 50016, the isolated mice were treated with dose of 3 mg/kg intraperitoneally and 0.5, 2, 4 and 5 h later, the latency time to attack was measured. An increase above the latency time observed for control (the latency time measured with 0.9% saline treatment) is interpreted as an anxiolytic effect of LB50016.

RESULTS

Ligand binding assay

As shown in Table II, LB50016 exhibited high selectivity and affinity for the 5-HT_{1A} receptor with Ki value of 2.7 nM for the displacement [^{3}H]8-OH-DPAT. This compound also binds to the 5-HT_{2A} receptor, adrenergic α_2 receptor and dopaminergic D_2 receptor with Ki values of 34, 16 and 195 nM, respectively. In addition to the binding at these receptors, LB50016 binds to dopaminergic D_1, muscarinic M_1 and M_2 receptors with two-order of magnitudes less affinities as compared with the 5-HT_{1A} receptor.

Table II. Affinity constants for LB50016

Receptors	Ki [nM]
5-HT_{1A}	2.7
5-HT_{2A}	34
Adrenergic α_2	16
Dopaminergic D_1	12,502
Dopaminergic D_2	195
Muscarinic M_1	857
Muscarinic M_2	706

Effects on serum corticosterone concentrations in rats

Fig. 2 demonstrates that oral (10 mg/kg) and intraperitoneal (3 mg/kg) applications of LB50016 induced about 3-folds and 1.5 to 2.5-folds increases in serum corticosterone concentrations as compared with saline-treated group, respectively. These effects are comparable to those observed with same doses of buspirone, respectively.

Effects on body temperature in rats

As shown in Fig. 3, LB50016 caused 1.5°C and 1.8°C reductions in rat rectal temperatures when measured at 30 and 60 min after subcutaneous administrations (3 mg/kg), respectively, which is comparable to those of buspirone at the same time points.

Effects on the forced swim test for antidepressant-like activity in mice

As shown in Fig. 4A, intraperitoneal treatment with LB50016 caused dose-dependent reductions in immobility time, where there were 31, 44 and 75% reductions as compared with control at 1, 3 and 5 mg/kg doses, respectively. Half-maximum effective dose (ED_{50}) is estimated to be 3 mg/kg. In order to characterize the specific receptors involved in the anti-immobility effects of LB50016, further pharmacological studies were performed through the receptor blockade experiments

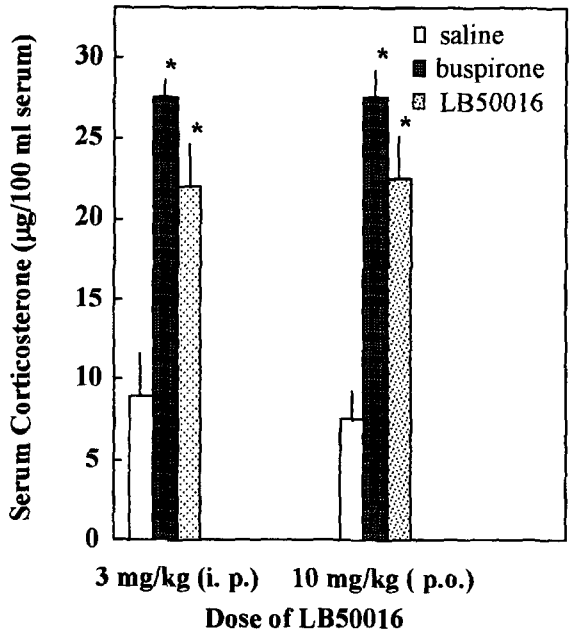

Fig. 2. Effects of LB50016 on the serum corticosterone concentration in rats. Animals were sacrificed 60 min after the test compounds were administered. The values represent the mean±S.E.M. for 6~8 male Sprague Dawley rats per dose. *denotes statistical significance ($p<0.05$) from control values.

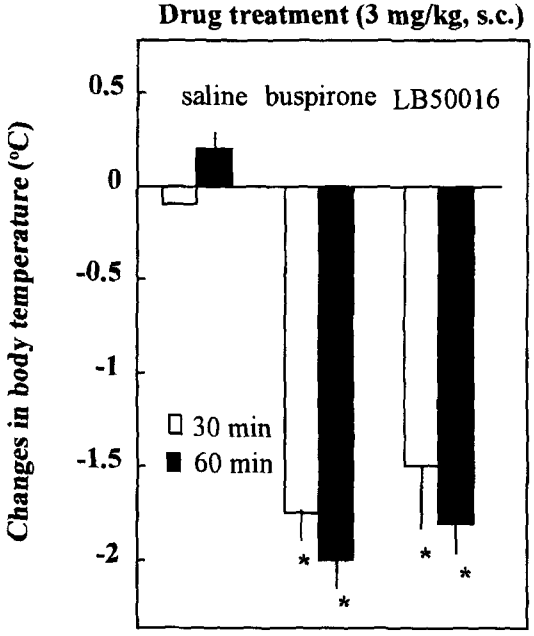

Fig. 3. Effects of LB50016 on the body temperature in rats. Temperature recordings were done as described in "Materials and methods". Each values represent the mean±S.E.M. for 6~8 male Sprague Dawley rats. *denotes statistical significance ($p<0.05$) from control values.

with NAN-190, a potent 5-HT_{1A} recetor antagonist. As shown in Fig. 4B, about 40% reduction in immobility time was obtained by intraperitoneal injection of LB 50016 with 3 mg/kg, while application of NAN-190 (3 mg/kg, i.p.) alone did not cause any reduction in immobility time as compared with control. However, pretreatment of mice with NAN-190 (3 mg/kg, i.p.) completely abolished the LB50016-induced inhibitory effect on immobilization, confirming that LB50016-mediated response was due to the 5-HT_{1A} receptor stimulation.

Effects on face-to-face test for anxiolytic activity in mice

Treatment of LB50016 and buspirone increased face-to-face interaction time in a dose-dependent manner up to about six-folds the level of saline-treated group. Estimated half maximal effective doses (ED_{50}) of LB 50016 and buspirone are approximately 2 mg/kg, i.p., respectively (Fig. 5).

Effects on isolation-induced aggression test in mice

Fig. 6 indicates that the treatment of LB50016 (3 mg/kg, i.p.) increase significant latency to attack, showing changes in it from 6 s (control) to 300 s, 175 s, 70 s, at 30, 120, 240 min after drug treatment, respectively. No effects were observed at 5 h after treatment.

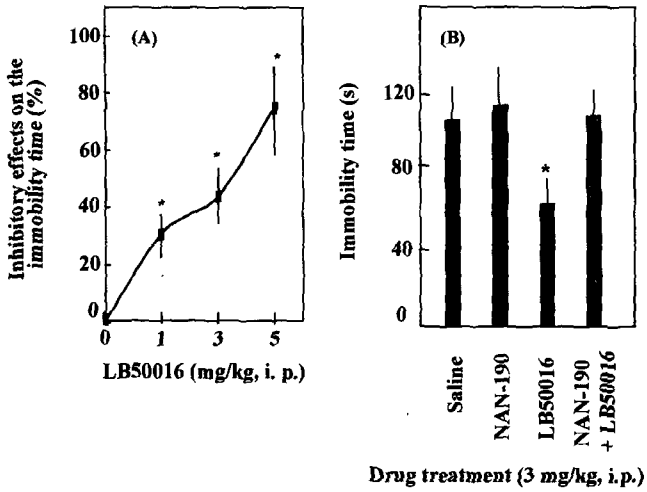

Fig. 4. (A) Dose-dependent effects of LB50016 on the forced swim test in mice. Various doses of LB50016 was injected intraperitoneally and immobility time was measured as described in "Materials and methods". Percent inhibitory effects were calculated as follows; [(immobility time of saline treated group-immobility time of drug treated group)÷immobility time of saline treated group]×100(%). Each values represent the mean±S.E.M. for 6~8 male ICR mice. *denotes statistical significance ($p<0.05$) from control values. (B) Effects of LB50016 in the pretreatment of NAN-190 on the forced swim test in mice. 3 mg/kg of NAN-190, a potent 5-HT_{1A} receptor antagonist was pretreated intraperitoneally 30 min before the application of LB50016 and the LB50016-induced alterations in the immobility time was measured as described in "Materials and methods". Each values represent the mean±S.E.M. for 8~10 male ICR mice. *denotes statistical significance ($p<0.05$) from control values.

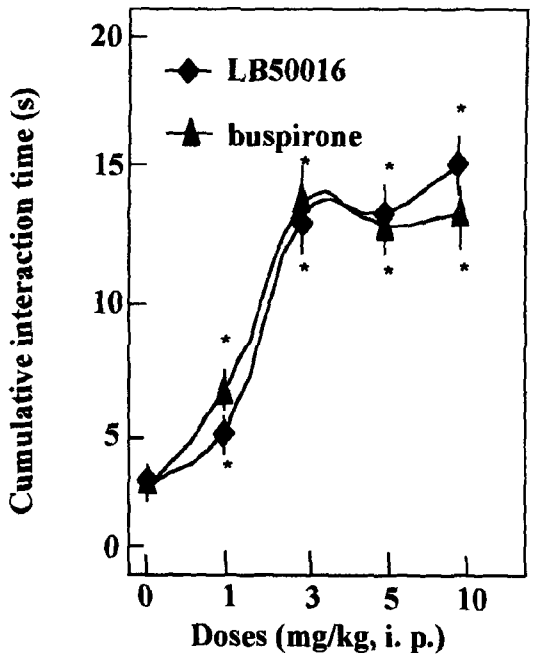

Fig. 5. Effects of LB50016 on the face-to-face test in mice. Thirty min after intraperitoneal administration of various doses of the test compounds, cumulative interaction time was measured as described in "Materials and methods". The values represent the mean±S.E.M. for 8~10 male ICR mice per dose.

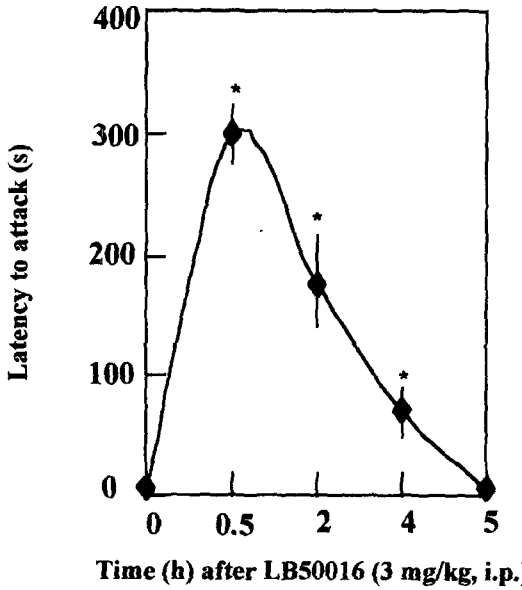

Fig. 6. Effects of LB50016 on the isolation-induced test in mice. At various time points after 3 mg/kg i.p. application of LB50016, the latency to attack was measured as described in "Materials and methods". Each values represent the mean±S.E.M. for 7~10 male ICR mice. *denotes statistical significance ($p<0.05$) from control values.

DISCUSSION

In vitro ligand binding and in vivo pharmacological studies were performed to characterize LB50016 as an 5-HT_{1A} receptor agonist and as a putative antidepressant and anxiolytic. Ligand binding profile of LB50016 (Table II) indicates that treatment of LB 50016 will preferentially occupy the 5-HT_{1A} receptor site with high affinity (Ki, 2.7 nM) and cause certain serotonin-like effects, while some other effects on the adrenergic α_2 and 5-HT_{2A} receptors are also expected to a certain extent. The well known 5-HT_{1A} receptor agonists 8-OH-DPAT, 5-MeO-DMT, buspirone and ipsapirone show more or less similar serotonergic receptor selectivity (Zifa and Fillion, 1992) to that of LB50016. The majority of their biological effects are considered to be exerted via 5-HT_{1A} receptor activation without considerable effects on the other receptor systems (Foreman *et al.*, 1994; McCall *et al.*, 1994; Scott *et al.*, 1994). Thus, most of the studies were performed to figure out effects of LB50016 in terms of the 5-HT_{1A} receptor-related characteristics. According to the publication, the collective profile of responses, so called 5-HT syndrome (flat body posture, reciprocal forepaw treading, side-to-side body movement, resting tremor, hind limb abduction and Straub tail) was used in order to characterize putative serotonergic com-

pounds. In addition to this, hypothermia and elevation in serum corticosterone concentration were suggested to be mediated by 5-HT_{1A} receptor activation. For example, direct injection of 6-OH-DPAT into the dorsal raphe nulei resulted in a reduction in temperature of rats and at least part of this response was proposed to be mediated by pre-synaptic 5-HT_{1A} receptors (Hillegaart, 1991). In contrast, other reports demonstrated that 5-HT_{1A} receptor-mediated hypothermic responses in the rats can be considered as an index of either pre- or post-synaptic 5-HT_{1A} receptor activation (Hutson *et al.*, 1987; Bill *et al.*, 1991). Several reports were documented on the issue of the hypothalamic-pituitary-adrenal axis for the control of corticosterone secretion. Serotonergic receptors induce release of the corticotropin-releasing hormone stimulating secretion of the adrenocorticotropic hormone, which in turn stimulates secretion of the glucocorticoids. This paradigm is currently believed to be mediated by post-synaptic 5-HT_{1A} receptors (Koening *et al.*, 1988; Fuller, 1990; Fuller and Snoddy, 1990). On the basis of these results, serotonergic properties of the LB50016 were characterized. For instance, 5-HT syndrome (flat body posture and hind limb abduction) known to be linked to 5-HT_{1A} receptor activation (Smith and Peroutka, 1986) were observed with the intraperitoneal application of LB50016 and buspirone at the dose of 3 mg/kg to the rats (unpublished observations). The hypothermic response and the elevation in serum corticosterone level induced by LB50016 and buspirone (Fig. 2 and 3) are consistent with the result from others experiments with buspirone and 8-OH-DPAT, respectively (Koenig *et al.*, 1988; Matheson *et al.*, 1997). Therefore, effects of LB50016 on hypothermia and serum corticosterone are supposed to be mediated by the 5-HT_{1A} receptor activation. Many of the previous reports suggest that there are certain relationships between biogenic amine deficiencies and affective disorders (anxiety and depression) and many drugs with the different mode of actions have been used to treat this disorders (Pinder and Wieringa, 1993). The clinical use of buspirone, 5-HT_{1A} receptor agonist in the treatment of generalized anxiety disorder and depression led us to test any *in vivo* efficacy of LB50016 in terms of antidepressant and anxiolytic (Napoliello and Domantay, 1991). Among the various models employed to date for the evaluation of potential antidepressant activity, the forced swim test is considered to have a high reliability (Porsolt *et al.*, 1977; Willner, 1984). In this model, experimental animals that are exposed to a forced swim stress rapidly decrease escape behavior, which is measured by an increased immobility time. Azapirone 5-HT_{1A} agonists such as buspirone, ipsapirone and gepirone, as well as other effective antidepressant have been reported to reduce immobility time (Wieland and Lucki, 1990). However, there are certain contradictory reports on these results, depending on the intrinsic efficacy of the test drugs. For example, 8-OH-DPAT, a full agonist on 5-HT_{1A} receptor shows a remarkable antidepressant efficacy in the forced swimming test (Gardner *et al.*, 1988; McCall *et al.*, 1994), while buspirone, known as a partial agonist revealed almost no efficacy (Abe *et al.*, 1996). One possible explanation for this is that 1-pp, a metabolite of buspirone having an affinity to the adrenergic α_2 receptor, blocks the antidepressant effects of parent drug. However, our results clearly show the LB50016-induced attenuation of immobile behavior (Fig. 4A). Additional confirmation of 5-HT_{1A} receptor-mediated inhibitory effects of LB50016 on immobility time was provided by the receptor blockade studies using NAN-190, a potent post-synaptic 5-HT_{1A} receptor antagonist, where LB50016-induced anti-immobilization effect is almost completely masked by the pretreatment of NAN-190 (Fig. 4B). Conclusively, this results show that LB50016 exerts its antidepressant activity at least in part by the stimulation of 5-HT_{1A} receptor. In order to substantiate anxiolytic efficacy of LB50016 in parallel with buspirone as a reference drug, face-to-face test, the frequently used candidate test for anxiolytic activity was carried out (File, 1980; Schreur, 1988; McCall *et al.*, 1994) and those drugs turn out to be effective with same efficacy (Fig. 5). In addition to this, LB50016-induced anxiolytic effect was further evaluated in the isolation-induced aggression test, another useful model to predict anxiolytic effects of test drugs (McCall *et al.*, 1994). Aggressive behavior was alleviated by the treatment of LB50016 with maximal effectiveness at 30 min after treatment (Fig. 6). These data combined with other LB50016-induced pharmacological activities described in the present study suggest that LB50016 has a 5-HT_{1A} agonist activity and may offer an effective therapeutic candidate in the management of anxiety and depression in humans.

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