NHBA isolated from *Gastrodia elata* exerts sedative and hypnotic effects in sodium pentobarbital-treated mice

Ying Zhang, Min Li, Rui-Xia Kang, Jian-Gong Shi, Geng-Tao Liu, Jian-Jun Zhang

**ABSTRACT**

The rhizomes of *Gastrodia elata* have been used for the treatment of insomnia in oriental countries. *N*-((4-hydroxybenzyl) adenine riboside (NHBA) was originally isolated from *G. elata*. For the first time we report a detailed study on the effects and mechanisms of NHBA on its sedative and hypnotic activity. Adenosine, an endogenous sleep factor, regulates sleep–wake cycle via interacting with adenosine A1/A2A receptors. Using radioligand binding studies and cAMP accumulation assays, our results show that NHBA may be a functional ligand for the adenosine A1 and A2A receptors. NHBA significantly decreases spontaneous locomotor activity and potentiates the hypnotic effect of sodium pentobarbital in mice. Sleep architecture analyses reveal that NHBA significantly decreases wakefulness time and increases NREM sleep times. However, NHBA does not affect the amount of REM sleep. Pretreatment with the adenosine A1 receptor antagonist DPCPX or the A2A receptor antagonist SCH 58261 significantly reverses the increase in sleeping time induced by NHBA in sodium pentobarbital-treated mice. Immunohistochemical studies show that NHBA increases c-Fos expression in GABAergic neurons of the ventrolateral preoptic area (VLPO), which suggests that NHBA activates the sleep center in the anterior hypothalamus. Altogether, these results indicate that NHBA produces significant sedative and hypnotic effects. Such effects might be mediated by the activation of adenosine A1/A2A receptors and stimulation of the sleep center VLPO.

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**1. Introduction**

Insomnia is an extremely common symptom both de novo and due to other medical and psychiatric disorders causing both physical and mental health impairments (Van Someren, 2000). However, hypnotics in clinical applications show many unpleasant reactions, such as drug dependence, tolerance, rebound insomnia and amnesia (Griffiths et al., 1986; Bocca et al., 1999). The search for novel pharmacotherapeutics for psychiatric illnesses from medicinal plants has progressed significantly in the past decade. A considerable number of herbal constituents whose behavioral effects and pharmacological actions have been well characterized may be good candidates for further investigations that may ultimately lead to clinical use (Zhang, 2004).

The rhizomes of *Gastrodia elata* have been extensively used in traditional Chinese medicine for the treatment of headaches, dizziness, vertigo, insomnia and convulsive illnesses, such as epilepsy and tetanus (Hus et al., 1986). Among its multiple indications, the sedative and hypnotic effects of *G. elata* have been shown in human and animal models (Yang et al., 2000). In modern pharmacological studies, *G. elata* contains many pharmacologically active ingredients, such as gadorstin, bis (4-hydroxybenzyl) sulfide and *N*-((4-hydroxybenzyl) adenine riboside (NHBA) (Guo et al., 1991; Huang et al., 2007). Among these ingredients, NHBA, an adenosine analog, can prevent serum deprivation-induced apoptosis in PC12 cells (Huang et al., 2007) and exert a beneficial effect on symptom progression in a mouse model of Huntington’s disease (Huang et al., 2011) by activating adenosine A2A receptor.

Adenosine, an endogenous sleep factor, regulates sleep–wake cycle via interacting with adenosine receptors. Adenosine A1 and A2A receptors are involved in the hypnogenic effect of adenosine through different brain areas and activation of adenosine A1/A2A receptor induces sleep. Conversely, caffeine, a nonspecific antagonist of adenosine receptors, is widely known for its capacity to reduce sleep and maintain wakefulness through adenosine A2A receptor (Fredholm et al., 1999; Huang et al., 2005; Lazarus et al., 2011). Because NHBA is one of the bioactive compounds of *G. elata* and an agonist of the A2A receptor (Huang et al., 2007, 2011), we investigated the sedative and hypnotic effects of NHBA and whether such effects are mediated by adenosine receptors.

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**Keywords:**
Gastrodia elata
Sleep
Adenosine receptors
Ventrolateral preoptic area
Insomnia
Hypnotics
2. Materials and methods

2.1. Animals

Adult male ICR mice (weighing 22 ± 2 g) and adult male Sprague-Dawley rats (weighing 260–280 g) were obtained from the Vital River Laboratories (Beijing, China). The animals were housed in acrylic cages (45 × 60 × 25 cm) with water and food available ad libitum under an artificial 12-h light/dark cycle (light from 8:00 a.m. to 8:00 p.m.) in a sound-proof room (22 ± 1 °C). To ensure adaptation to their new environment, they were kept in the departmental holding room for one week before testing. Experiments were carried out in compliance with the National Institutes of Health and institutional guidelines for the humane care of animals and were approved by the Animal Care Committee of the Peking Union Medical College & the Chinese Academy of Medical Sciences. Every effort was made to minimize the number of animals used and any pain and discomfort experienced by the subjects.

2.2. Drugs

NHBA (Fig. 1, purity >99%) was synthesized by Professor Jiangong Shi, Department of Phytochemistry of the Institute of Materia Medica, Beijing, China. NHBA was identified by comparing its physical and chemical properties with published data (Huang et al., 2007). Other drugs used in this study are [3H] 8-cyclpentyl-1,3-dipropylxanthine ([3H] DPCPX, Radiolabeled Chemicals), [3H] 3-(3-hydroxypropyl)-7-methyl-8-(m-methoxy styryl)-1-propargylxanthine ([3H]MSX-2, Radiolabeled Chemicals), rolipram (Sigma), cAMP (Sigma), [3H]cAMP (Perkin-Elmer), SCH 58261 (Tocris, Radiolabeled Chemicals), Radiolabeled Chemicals), [3H] 3-(3-hydroxypropyl)-7-methyl-8-(m-methoxy styryl)-1-propargylxanthine ([3H]MSX-2, Radiolabeled Chemicals), rolipram (Sigma), cAMP (Sigma), [3H]cAMP (Perkin-Elmer), pentobarbital sodium (China National Pharmaceutical Group Corporation), zolpidem (ZPD, Sigma), DPCPX (Tocris), and 5-amino-7-(β-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261, Tocris). The rabbit polyclonal anti-αs-Fos and rabbit polyclonal anti-c-Fos antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz). The VectaStain® Elite ABC kit and rabbit polyclonal anti-GAD-67 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz). The VectaStain® Elite ABC kit and rabbit polyclonal anti-GAD-67 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz).

2.3. Treatments

For intraperitoneal (i.p.) injection (0.1 ml/10 g), sodium pentobarbital was dissolved in physiological saline. NHBA, zolpidem, DPCPX and SCH 58261 were suspended in 1% Tween 80/physiological saline. Sodium pentobarbital was dissolved in physiological saline. NHBA, zolpidem, DPCPX and SCH 58261 were suspended in 1% Tween 80/physiological saline.

2.4. Receptor binding and functional assays

2.4.1. Cell culture and membrane preparation

2.4.1.1. Cell culture. CHO (Chinese hamster ovary) cells expressing the recombinant human A1/A2A receptors (provided by Professor R. B. Su of Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences) were cultured in Dulbecco’s Modified Eagles Medium with the F12 nutrient mixture (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (2 mM) and Geneticin at 37 °C in 5% CO2/95% air. The cells utilized for cAMP determinations had a viability >95%, as assessed by trypan blue exclusion.

2.4.1.2. Membrane preparation. Male Wistar rats were sacrificed. The cerebral cortex and striatum were removed and homogenized in 10 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenates were centrifuged (48,000 g) at 4 °C for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellets were resuspended in 50 mM Tris–HCl buffer containing 1.2 μg per mg protein of adenosine deaminase to remove endogenous adenosine.

2.4.2. Binding assays at the A1 and A2A receptors

NHBA was investigated in radioligand binding assays at A1 adenosine receptors of rat brain cortical membranes using 0.2 nM [3H] DPCPX (85 Ci/mmol) and at A2A adenosine receptors of rat striatal membranes using 0.75 nM [3H] MSX-2 (104.8 Ci/mmol). The binding assays were carried out in Tris–HCl buffer (50 mM, pH 7.4) as previously described (Klotz et al., 1989; Müller et al., 2000). Briefly, assays were performed by incubating the mixtures on a shaking water-bath at 25 °C for 30 min. Termination of the incubation was performed by rapid filtration through GF/B glass fiber filters. The radioactivity on the filter was measured by a scintillation counter. The nonspecific binding was defined as the binding activity in the presence of 10 μM of 2-chloroadenosine (2-CADO) or 50 μM NECA for adenosine A1 and A2A binding assays, respectively. In saturation analysis the KD value was 0.14 nM and the Bmax value was 2290 fmol/mg protein for binding of [3H]DPCPX to the A1 adenosine receptor. Meanwhile, the KD value was 11.48 nM and the Bmax value was 5657 fmol/mg protein for binding of [3H]MSX-2 to the A2A adenosine receptor. Inhibition curves were determined using eight different concentrations ranging from 10−5 to 10−8 M (Uhlén et al., 1998). The inhibition constants (Ki) were calculated using the equation of Cheng and Prusoff (1973).

2.4.3. Cyclic AMP accumulation assay

Intracellular cAMP levels were measured with a competitive protein binding method (Nordstedt and Fredholm, 1990). For the measurement of A1/A2A receptor-mediated accumulation of cAMP, cells were grown as confluent monolayers in 24-well cluster dishes. The cells were washed twice with 2 ml of medium containing 20 mM HEPES pH 7.4 prewarmed to 37 °C. The cells were then preincubated in 0.4 ml fresh medium containing the 30 μM of the cAMP phosphodiesterase inhibitor rolipram for 15 min. NHBA was added in a volume of 0.1 ml medium and incubations were continued for 15 min. The reaction was terminated by removing the supernatant, and cells were lysed with the addition of 500 μl of 0.1 M ice-cold HCl. The cell lysate was centrifuged (4000 g) at 4 °C for 10 min. The supernatants or cAMP standards (0–8 pmol) were incubated with [3H]cAMP (27 Ci/
mmol) and cAMP binding protein in 96-well microtiter plates at 4 °C for 150 min. Free and bound [3H]cAMP were separated by filtration over Whatman GF/B filters using a semi-automatic cell harvester. Each filter was rinsed with 3 ml of 50 mM Tris–HCl, pH 7.4. Bound radioactivity was measured by liquid scintillation spectrometry.

2.5. Behavioral analyses

2.5.1. Spontaneous locomotor assay

The sedative property of NHBA was evaluated using locomotor activity as an index. Spontaneous locomotor activity was determined in ICR mice which were placed in a transparent chamber (25 cm diameter × 13 cm height) that connected to an automatic registration system (ZIL-2, Institute of Materia Medica, China). The chamber was equipped with six infrared sensors arranged along the bottom of the wall of the arena. Twenty minutes after treatment with NHBA (0.2, 1 and 5 mg/kg, i.p.), zolpidem (2 mg/kg, i.p.) or 1% Tween 80, mice were placed in the test chamber. The interruptions of beams of two consecutive infrared sensors were collected for 5 min as a reflection of locomotor activity. After each testing session, the enclosures were thoroughly cleaned with 70% ethanol and water.

2.5.2. Evaluation of the latency and the duration for loss of righting reflex

Experiments were carried out between 1:00 p.m. and 5:00 p.m. following sodium pentobarbital injection, each mouse was observed for the onset of sleep. When the mice lost the righting reflex for over 1 min, they were considered to be asleep. The loss of righting reflex was defined as a failure of the mouse to right itself for at least 10 s after being placed on its back. Time elapsed between the administration of sodium pentobarbital and the loss of righting reflex was recorded as the latency for loss of righting reflex, while the time from the loss of righting reflex to recovery was recorded as the duration for loss of righting reflex (Soulimani et al., 2001). In the sub-hypnotic dosage of sodium pentobarbital treatment test, the percentage of sleep onset was calculated as follows: Sleep onset (%) = No. falling asleep/Total no. × 100%.

2.5.3. EEG recording

Under deep anesthesia with sodium pentobarbital (45 mg/kg, i.p.), mice were chronically implanted with electrodes as described in detail elsewhere (Clément et al., 2005). Briefly, three stainless-steel screws (one frontal: 1 mm anterior and 1 mm lateral to the bregma; one parietal: 2 mm posterior and 1 mm lateral to the bregma; one cerebellar: 1 mm posterior and 1 mm lateral to lambda according to the Paxinos and Watson atlas) were implanted to record the electroencephalogram (EEG). Two stainless-steel wires were inserted into the neck muscle to record the electromyogram (EMG). The connecting wires coming from the electrodes were soldered to a six-pin connector and anchored to the skull with dental cement. After surgery, the mice were housed singly and allowed one week to recover.

EEG and EMG recordings were performed immediately after the injection of NHBA (1 and 5 mg/kg) or vehicle. The signals were amplified and sampled at 200 Hz by a polygraph (Model MP100, BIOPAC, CA, USA). The signals were filtered (EEG, 0.5–30 Hz; EMG, 16–128 Hz), then digitized at a sampling rate of 128 Hz and recorded using AcqKnowledge software (BIOPAC systems, Inc. USA). Technicians blind to the treatment scored the EEG and EMG data through 4-s epochs for wake, NREM sleep and REM sleep according to the standard sleep architecture criteria (Robert et al., 1999). Briefly, each state was characterized as follows: wake, low amplitude high frequency EEG and high-voltage EMG activities; NREM sleep, high amplitude slow/low-frequency (0.5–4 Hz) synchronous pattern or spindle EEG and low EMG activities; REM sleep, low-voltage EEG featured theta wave (4–8 Hz) and very low EMG activities. Recordings were performed during the normal wake period of mice to have better observation of the hypnotic effect of NHBA. The analysis was restricted to 3 h after injections.

2.6. c-Fos immunohistochemistry

Two groups of rats were used. One group was treated with vehicle while the other group was intraperitoneally injected with 5 mg/kg NHBA. At 2 h after NHBA administration, the animals were anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused via the heart with saline solution followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Their brains were then fixed with chloral hydrate (350 mg/kg, i.p.) and perfused via the heart with saline solution followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Their brains were then fixed with chloral hydrate (350 mg/kg, i.p.) and perfused via the heart with saline solution followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Their brains were then fixed with chloral hydrate (350 mg/kg, i.p.) and perfused via the heart with saline solution followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4).

Sections were double-immunostained using previously described methods (Sherin et al., 1996, 1998; Elmquist et al., 1996). Sections were counterstained for glutamic acid decarboxylase (GAD; rabbit polyclonal antibody; 1:400, Santa Cruz) using goat anti-rabbit IgG polymer (ABC kit) and visualized using DAB without nickel for brown staining. Sections were then rinsed twice in 3% hydrogen peroxide for 10 min to quench the endogenous peroxidase activity. Sections were then rinsed twice in 10 mM phosphate-buffered saline (PBS, pH 7.2) containing 0.3% Triton X-100. This was followed by incubation with diluted normal blocking serum (Vector Elite ABC kit) for 30 min and with 1:400 rabbit polyclonal anti-c-Fos (primary) antibodies overnight at 4 °C. Biotinylated secondary antibody solutions and VectaStain® Elite ABC reagents were applied. The peroxidase reaction was visualized using DAB with nickel ammonium sulfate for black staining. Next sections were counterstained for glutamic acid decarboxylase (GAD; rabbit polyclonal antibody: 1:400, Santa Cruz) using goat anti-rabbit IgG and visualized using DAB without nickel for brown staining. Sections were mounted, dehydrated and cover slipped. As controls, adjacent sections were incubated without the primary antibody to confirm that no non-specific staining had occurred.

Using light microscopy (Nikon Eclipse 80i), c-Fos positive neurons were identified by dense black nuclear staining and GAD-positive neurons in the VLPO were identified by brown cytoplasmic staining. Locations in the brain were confirmed by delineation of the Paxinos

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Fig. 2. The competitive curves of the binding assay for NHBA at A1 adenosine receptor (A) and A2A adenosine receptor (B).
and Watson rat atlas. Four sections through the middle of each structure per animal were counted (blind to treatment) and averaged.

2.7. Statistical analysis

The results were expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by Newman Keuls post-test for multiple comparisons. For the sub-hypnotic dosage of pentobarbital sodium induced sleeping test, Fisher’s exact test was used to compare the rate of sleep onset between the control and each of the other groups. The expression of c-Fos in the VLPO was analyzed by the unpaired t-test. Differences were considered statistically significant at P<0.05.

3. Results

3.1. Pharmacological properties of NHBA

We characterized the pharmacological properties of NHBA using radioligand binding assays. NHBA was tested for its ability to displace [3H]DPCPX from A1 adenosine receptor in rat cortical membranes and [3H]MSX-2 from A2A adenosine receptor in striatal membranes, respectively. Adenosine A1 and A2A receptor binding affinities for NHBA are expressed as Ki values. In the A1 receptor binding assay NHBA had a Ki value of 1.39 μM (Fig. 2A), whereas in the A2A receptor binding assay NHBA had a Ki value of 1.39 μM (Fig. 2B). Data showed that NHBA bound to adenosine A1 and A2A receptors with moderate affinity.

![Fig. 3. The dose response curves for the cAMP assay of NHBA. Inhibition of forskolin-stimulated cAMP production by NHBA or CPA in CHO cells expressing human adenosine A1 receptor (A). The accumulation of forskolin-stimulated cAMP by NHBA or NECA in CHO cells expressing human adenosine A2A receptor (B).](image)

The modulation effects of NHBA on adenosine A1 and A2A receptors were studied by measuring cAMP accumulation in CHO cell lines stably transfected with recombinant human adenosine A1 or A2A receptors. Activation of A1 receptor inhibits adenylyl cyclase activity and subsequently decreases cAMP level whereas activation of A2A receptor produces the opposite effects (Londos et al., 1980; Van Calker et al., 1979; Kenakin, 1996). Our results showed that NHBA decreased the cellular cAMP level in CHO cells stably expressing the A1 receptors with EC50 of 0.96 μM (Fig. 3A), whereas NHBA elevated cAMP levels in CHO cells expressing A2A receptors with EC50 of 0.0675 μM (Fig. 3B). These data indicate that NHBA is a functional ligand for the adenosine A1 and A2A receptors.

3.2. Behavioral changes by administration of NHBA

The sedative activity of NHBA was investigated by recording the spontaneous locomotor activity of mice. Compared with the control group, the positive control zolpidem (2 mg/kg) induced a significant reduction in spontaneous locomotor activity (P<0.001). Intraperitoneal administration of NHBA (0.2, 1 and 5 mg/kg) significantly decreased locomotor activity by 63%, 68% and 79%, respectively (F(4,36) = 10.45; P<0.001) (Fig. 4).

The hypnotic activity of NHBA was investigated in sodium pentobarbital treated mice. NHBA (0.2, 1 and 5 mg/kg, i.p.) significantly increased the rate of sleep onset in mice treated with a sub-hypnotic dose of sodium pentobarbital (22 mg/kg) (Table 1). Zolpidem (2 mg/kg, i.p.), the positive control used in this study, also significantly potentiated the sodium pentobarbital hypnosis.

NHBA significantly potentiated the effects of the hypnotic dose of sodium pentobarbital. Pretreatment of NHBA (0.2, 1 and 5 mg/kg, i.p.) for 20 min significantly shortened the latency for loss of righting reflex by 33%, 63% and 53%, respectively (F(4,37) = 4.51; P<0.001) (Fig. 5A) and prolonged the duration for loss of righting reflex by 10%, 79% and 150%, respectively (F(4,37) = 11.48; P<0.001) (Fig. 5B), compared with the control group. As expected, zolpidem (2 mg/kg, i.p.) significantly potentiated the sodium pentobarbital induced sleep in mice.

![Table 1. The effect of NHBA on the sleep onset of mice treated with a sub-hypnotic dose of sodium pentobarbital. Twenty minutes after administration of zolpidem (ZPD, 2 mg/kg, i.p.) and NHBA (0.2, 1 and 5 mg/kg, i.p.), sodium pentobarbital (22 mg/kg, i.p.) was given to mice. The number of mice falling asleep was recorded. *P<0.05, **P<0.01 and ***P<0.001 vs. control group (Fisher’s exact test).](image)
3.3. Role of adenosine receptor in the hypnotic effect of NHBA

3.3.1. Counteraction of the selective adenosine A1 receptor antagonist DPCPX on the hypnotic activity of NHBA

DPCPX (0.2 and 0.5 mg/kg, i.p.), a selective A1 receptor antagonist, showed no significant effect on the duration for loss of righting reflex in mice treated with the hypnotic sodium pentobarbital (40 mg/kg, i.p.). When used in combination with NHBA, 1 mg/kg DPCPX partially antagonized the increase in the duration for loss of righting reflex induced by NHBA \( F(5,60) = 3.80; P < 0.05 \) (Fig. 6).

3.3.2. Counteraction of the selective adenosine A2A receptor antagonist SCH 58261 on the hypnotic activity of NHBA

SCH 58261 (0.2 and 1 mg/kg, i.p.), a selective A2A receptor antagonist, showed no significant effect on the duration for loss of righting reflex induced by the hypnotic dose of sodium pentobarbital (40 mg/kg, i.p.). When used in combination with NHBA (1 mg/kg, i.p.), SCH 58261 significantly antagonized the NHBA-induced increase in the duration for loss of righting reflex \( F(5,60) = 4.50; P < 0.05 \) (Fig. 7).

3.4. The effect of NHBA on the sleep architecture in free-moving mice

The sleep-promoting activity was confirmed by EEG analysis in free moving mice. NHBA (1, 5 mg/kg) significantly prolonged NREM sleep \( F(2, 15) = 20.02; P < 0.001 \) and shortened wakefulness \( F(2, 15) = 15.63; P < 0.001 \) during the first hour following drug administration, compared with the vehicle group (Fig. 8A). During the second hour following drug administration, the significant increase in NREM sleep \( P < 0.05 \) and decrease in wakefulness \( P < 0.05 \) was maintained in the 5 mg/kg NHBA-treated group. However, a trend of decreasing NREM sleep and increasing wakefulness was observed in the 1 mg/kg NHBA-treated group, indicating a 'rebound' arousal effect. The effect of NHBA on vigilance states finally dissipated during the third hour (Fig. 8C). There was no significant suppression of REM sleep following NHBA administration.

3.5. NHBA increased c-Fos expression in the VLPO

To study the effect of NHBA on the VLPO sleep center, we counted the number of c-Fos+GAD double-labeled neurons in the VLPO. Fig. 9
shows representative photomicrographs of the VLPO of rats treated with vehicle or NHBA. GAD-positive neurons stain brown in their cytoplasms. C-Fos positive neurons show black staining in their nuclei. GAD is a marker of GABAergic cells. C-Fos expression is a cellular marker for neuronal activity. NHBA at 5 mg/kg significantly increased C-Fos expression in the GABAergic neurons of the VLPO 2.3-fold (P<0.001), as compared with the vehicle control (Fig. 9D). These results indicate that NHBA activates the VLPO sleep center.

4. Discussion

To our knowledge, this is the first study to demonstrate that NHBA, one of the bioactive compounds of G. elata, exerts hypnotic actions on mice by simultaneously activating the adenosine A1 and A2A receptors.

The main approach for discovering adenosine receptor agonists has been modification of adenosine itself, and the structure–activity relationships of adenosine at adenosine receptors have been extensively probed (Yan et al., 2003). Most of the useful analogs are modified in the N6- or 2-position of the adenine moiety and in the 3′-, 4′- or 5′-position of the ribose moiety (Kim et al., 2003; Tchilibon et al., 2005). Agonist selectivity for A1 adenosine receptors is typically accomplished through substitution at the adenine N6-position, giving rise to compounds such as CPA. DPMA, a potent agonist for the A2A adenosine receptors, is also a N6-substituted adenosine derivative (Jacobson and Gao, 2006). NHBA is a N6-substituted adenosine derivative and Huang et al. (2011) demonstrated that NHBA is a novel agonist for adenosine A2A receptor. Our results indicate that NHBA is an agonist for both of the adenosine A1 and A2A receptors. The discrepancy on the affinity of NHBA at A1 adenosine receptor between the present study and Huang et al.’s study (2011) may be due to the different experimental methods. Further study is in progress to clarify the discrepancy.

Adenosine is an endogenous sleep substance. Adenosine and stable adenosine analogs induce sleep when administered to rats, cats and other experiment animals (Basheer et al., 2004). Adenosine affects cell functions through several mechanisms, but since only the membrane receptors are affected at physiological concentrations, receptor activation is the main way adenosine affects cell functions (Fredholm et al., 1999). There are four subtypes of adenosine receptors expressed in the central nervous system: A1, A2A, A2B and A3 (Fredholm et al., 2001). Because of their expression profiles and affinities toward adenosine, the A1 receptor and A2A receptor likely regulate important physiological functions in the brain (Yang et al., 2000). Several lines of evidence indicate that both A1 receptor and A2A

Fig. 8. Effects of NHBA on sleep architecture in free-moving mice. After administration of NHBA (1, 5 mg/kg, i.p.) or vehicle, EEG and EMG recordings were immediately measured for 3 h. Data are represented as the mean±S.E.M. (n=4–6). *P<0.05 and ***P<0.001, compared with the control group (one-way ANOVA followed by Newman Keuls post-test).

Fig. 9. The effect of NHBA on c-Fos expression in the VLPO. Photomicrographs of neurons in the VLPO that are GABAergic (brown cytoplasm, expressing the synthesizing enzyme GAD) and express the c-Fos protein (black nucleic stain). Illustration of the VLPO localization in the rat brain (A). A large scale photo shows the position of the VLPO in vehicle-treated and NHBA-treated rat brain (B and C). Arrowheads indicate example of double-labeled cells (F). Examples of c-Fos+GAD double-labeled cells in the VLPO of vehicle-treated (D) and NHBA-treated rats (E). Graphic depiction of the effect of NHBA (5 mg/kg, i.p.) on c-Fos expression in the VLPO (G). Values are expressed as the means±S.E.M. (n=4). ***P<0.001, compared with the control group (Student’s t-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
post-synaptic potentials (IPSPs) in rat VLPO neurons recorded in 456 Y. Zhang et al. / Pharmacology, Biochemistry and Behavior 102 (2012) 450
suggest that the adenosine A1 and A2A receptors are involved in the
prevented the NHBA-induced increase in sleeping time. Our results
excitability of the central nervous system (Masur et al., 1971). The
is considered an index of alertness and a decrease in motor activity
been evaluated. Therefore we characterized the sedative and hypnotic
property of NHBA.
The classic methods of spontaneous locomotor activity test and
pentobarbital induced sleeping tests are often used to screen seda-
tive–hypnotic drugs (Masur et al., 1971). Spontaneous motor activity
is considered an index of alertness and a decrease in motor activity
leads to sedation (Öztürk et al., 1996) as a result of the reduced
excitability of the central nervous system (Masur et al., 1971). The
sedative effects of drugs can be evaluated by measurement of pento-
barbital induced sleeping time in laboratory animals. The present
study showed that NHBA significantly decreased locomotor activity
and potentiated the hypnotic effect of sodium pentobarbital, indicat-
ing its central nervous depressant activity. Moreover, sleep
architecture analyses revealed that NHBA significantly shortened
wakefulness and prolonged NREM sleep and had no remarkable inhi-
bition of REM sleep. Both increasing NREM sleep and assuring the
normal amount of REM sleep are important for a sedative–hypnotic
drug to improve sleep effectively and enhance the quality of sleep
(Clément et al., 2005; McCarley, 2007). These effects differ from
those of diazepam, a common benzodiazepine derived drug pre-
scribed for insomnia (Tobler et al., 2001). Therefore, these results
demonstrate that NHBA can induce sleep and suggest its potential
use for the treatment of insomnia. Subsequently we used selective
A1/A2A receptor antagonists to investigate whether the hypnotic ef-
fect of NHBA was mediated by these receptors. When DPCPX or SCH 52621 was used in combination with NHBA, they significantly
prevented the NHBA-induced increase in sleeping time. Our results
suggest that the adenosine A1 and A2A receptors are involved in the
hypnotic effect of NHBA. Although the binding affinities of NHBA
were not as strong as those of the best adenosine drugs currently
available, the ability of NHBA to simultaneously activate adenosine
A1 and A2A receptors allows NHBA to effectively activate the
adenosinergic system to induce sleep.

The VLPO is a region of the mammalian hypothalamus that plays a
major role in the promotion of sleep (Sherin et al., 1996; Saper et al.,
2005; Gaus et al., 2002). The VLPO sends inhibitory GABAAergic and
galaninergic projections to multiple arousal systems in the brainstem
and hypothalamus, thus promoting sleep (Lu et al., 2002a,b; Chou
et al., 2005; Gaus et al., 2002). The VLPO sends inhibitory GABAergic and
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Monitoring adverse events and exploring its detailed hypnotic mech-
nisms should be conducted to evaluate a safe use of NHBA in the
future.

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