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In vitro and antinociceptive profile of HON0001, an orally active NMDA receptor NR2B subunit antagonist

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Abstract

The analgesic activity and side effect liabilities of a novel NR2B antagonist, 7-hydroxy-6-methoxy-2-methyl-1-(2-(4-(trifluoromethyl)phenyl) ethyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride (HON0001) were investigated. HON0001 inhibited [^{3}H]MK-801 binding to rat brain membranes in a biphasic manner, with IC_{50} values of 54.68±4.96 nM and 46.48±5.85 μM for high- and low-affinity sites, respectively. HON0001 inhibited [^{3}H]ifenprodil binding to membranes of rat cerebral cortex with an IC_{50} value of 57.01±3.4 nM, consistent with the results obtained for high-affinity sites of [^{3}H]MK-801 binding. HON0001 exhibited no or negligible affinity for other receptors, transporters and ion channels, while HON0001 had a moderate agonistic activity at μ-opioid receptors and affinity for dopamine D_1 receptors. HON0001 exhibited an analgesic effect in carrageenan-induced mechanical hyperalgesia and in the Seltzer model of partial sciatic nerve ligation following oral administration. In contrast, unlike MK-801, HON0001 did not affect spontaneous locomotor activity, rotarod performance and step-through latency in a passive avoidance task even at doses much higher than antinociceptive doses. HON0001 exhibited excellent brain penetration with a brain-to-plasma ratio of 34.5. These findings show that HON0001 is an orally active NR2B antagonist and that it may be useful for treating patients with neuropathic and other conditions without causing the side effects often observed with currently available non-subtype selective NMDA receptor antagonists.

Keywords: NR2B; HON0001; Neuropathic pain; Inflammatory pain

1. Introduction

Injury to a peripheral tissue or nerve often results in a neuropathic pain condition which is characterized by spontaneous pain, allodynia and hyperalgesia. These neuropathies may be persistent and are problematic because they are often poorly managed by conventional opioid analgesics and non-steroidal anti-inflammatory drugs. Neuropathic pain is not only due to an increase in the sensitivity of primary afferent nociceptors at the site of injury, but also depends on *N*-methyl-D-aspartate (NMDA) receptor-mediated central changes in synaptic excitability (Liu et al., 1998; Liu and Sandkuhler, 1998).

Recent attention has focused on NMDA receptor antagonists for the treatment of neuropathic pain. Indeed, ketamine, a non-selective NMDA receptor antagonist, has been reported to produce symptomatic relief in a number of neuropathies (Max et al., 1995). However, although NMDA receptor antagonists have been proven to be efficacious for various types of experimental and clinical pain situations, their use as analgesics is limited by serious side effects including psychotomimetic symptoms and effects on learning and memory (Balster and Willetts, 1996; Boyce et al., 1999). One promising approach to obtain safer analgesics includes subtype-selective NMDA receptor antagonism.

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The NMDA receptor is a ligand-gated ion channel composed of multiple subunits which are classified into two families: the NR1 subunit, of which there are at least eight splice variants (NR1a–h) and the NR2 subunit which has four subtypes (NR2A–D) each encoded by a separate gene (Mori and Mishina, 1995; Nakanishi, 1992). In rodent and human brain, the NR1 subunit is widely distributed (Nakanishi, 1992), while NR2 subunits display regional heterogeneity in expression. The predominant NR2 subunits in the forebrain are NR2A and NR2B, with NR2C expressed mainly in the cerebellum. NR2D expression is confined to the diencephalons and midbrain (Wenzel et al., 1995).

The heterogeneity of NMDA receptor subtype distribution in the central nervous system (CNS) has encouraged the development of subtype-selective compounds, with the possibility that such compounds may lack the side effects observed with nonselective NMDA receptor antagonists. Accumulating evidence has indicated the pivotal roles of NR2B receptors in pain perception. NR2B is located in the forebrain and laminas I and II of the dorsal horn (Boyce et al., 1999). It has been reported that rapid and prolonged increase in tyrosine phosphorylation of the NR2B subunit, but not the NR2A subunit, in the spinal cord was observed during the development and maintenance of inflammatory hyperalgesia, which may cause long-lasting NMDA receptor activation (Guo et al., 2001). Mice overexpressing the NR2B subunit in the anterior cingulate and insular cortices exhibit enhanced persistent pain and allodynia (Wei et al., 2001). Moreover, ifenprodil, a selective NR2B antagonist, has been shown to have antinociceptive effects at doses that do not impair motor performance (Boyce et al., 1999; Chizh et al., 2001), suggesting that this class of NMDA receptor antagonists may have a wider therapeutic window than nonselective NMDA receptor antagonists. This hypothesis was further supported by the findings that more potent and selective NR2B antagonists such as CP101,606 and Ro 63-1908 have wider therapeutic windows than ifenprodil in suppressing hyperalgesia in animal models of chronic pain and lack CNS side effects (Boyce et al., 1999; Taniguchi et al., 1997).

Recently, we synthesized a new NR2B antagonist, 7-hydroxy-6-methoxy-2-methyl-1-(2-(4-(trifluoromethyl)phenyl)ethyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride (HON0001) based on the structural feature of ifenprodil. Here, we describe the in vitro and in vivo antinociceptive profile of HON0001.

2. Materials and methods

2.1. Animals

Male Wistar rats (160–200 g, Charles River, Japan) were used for both in vitro and in vivo studies. All these animals were maintained under a 12-h light/dark cycle (light on 7:00 AM) in a temperature- and humidity-controlled holding room. Food and water were available ad libitum. All studies were reviewed by the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research Association standards, as defined in the *Guidelines for Animal Experiments* (1987).

2.2. Chemicals

7-Hydroxy-6-methoxy-2-methyl-1-(2-(4-(trifluoromethyl) phenyl)ethyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride (HON0001; Fig. 1) was synthesized in the Department of Drug Manufacturing Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University. [^{3}H]MK-801 (specific radioactivity: 1069.3 GBq/mmol), [^{3}H]ifenprodil (specific radioactivity: 1498.5 GBq/mmol), [^{3}H]DAMGO (specific radioactivity: 1887 GBq/mmol) and [^{35}S]GTPγS (specific radioactivity: 46.3 TBq/mmol) were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Ifenprodil, *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride (*R*(+)-3-PPP), 1-(2-[bis(4-Fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12909), 1-[2-(Diphenylmethoxy) ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12935) and carrageenan were purchased from Sigma-Aldrich (St. Louis, MO). Prazosin hydrochloride was from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of the highest purity available. For in vivo studies, HON0001 was dissolved in saline.

2.3. [^{3}H]MK-801 binding to rat brain membranes

[^{3}H]MK-801 binding was performed according to a method previously described with a minor modification (Fischer et al., 1997). Rat whole brain was homogenized with 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM EDTA, and centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was then centrifuged at 48,000×*g* for 20 min at 4 °C. The pellet was suspended in 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM EDTA, and incubated at 37 °C for 10 min, after which the pellet was washed with 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM EDTA by resuspension and recentrifugation. The pellet obtained was then suspended in 5 mM Tris–HCl buffer (pH 7.4) containing 1 nM glutamate and 1 nM glycine, at a protein concentration of 200 μg/ml, and served as a crude membrane preparation. Membrane preparation was inoculated with [^{3}H]MK-801 (3 nM) for 2 h at 25 °C. The reaction was terminated by rapid filtration through a Whatman GF/B glass

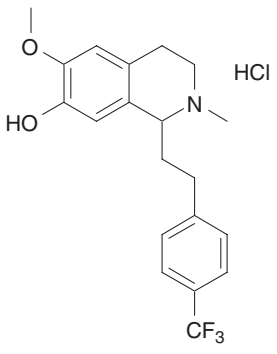

Fig. 1. Chemical structure of HON0001.

fiber filter presoaked with 0.3% polyethyleneimine for 2 h, after which the filters were washed three times with 3 ml of ice-cold 5 mM Tris–HCl buffer (pH 7.4), using an M-24R multi cell harvester (Brandel Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). Filter-bound radioactivity was counted in a liquid scintillation spectrometer (LS6000TA, Beckman Instruments Inc., Fullerton, CA). Nonspecific binding was determined in the presence of 100 μM ifenprodil.

2.4. [^{3}H]Ifenprodil binding to rat cerebral cortical membranes

[^{3}H]Ifenprodil binding assay was performed using a previously reported method with some modifications (Grimwood et al., 2000). Rat cerebral cortex was homogenized with 50 mM Tris-Acetate buffer (pH 7.0), and centrifuged at 48,000×*g* for 20 min at 4 °C. The pellet was homogenized and incubated for 30 min at 37 °C, and washed twice with 50 mM Tris-Acetate buffer (pH 7.0) by resuspension and recentrifugation. The final pellet was suspended in 50 mM Tris-Acetate buffer (pH 7.0) containing 100 μM *R*(+)-3-PPP (σ receptor blocker), 1 μM GBR-12909 (dopamine uptake inhibitor and μ receptor blocker), 1 μM GBR-12935 (dopamine uptake inhibitor and piperazine acceptor site blocker), and 10 μM prazosin hydrochloride (α1 adrenoceptor blocker), at a protein concentration of 350 μg/ml. Membrane preparation was incubated with 10 nM [^{3}H]ifenprodil for 1 h on ice. Nonspecific binding was determined in the presence of 100 μM ifenprodil.

2.5. [^{3}H]DAMGO binding to rat brain membranes

Rat whole brain (except cerebellum) was homogenized with 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 1 mM EDTA, and centrifuged at 48,000×*g* for 20 min at 4 °C. The pellets, washed once with the buffer, were suspended in 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 1 mM EDTA at a protein concentration of 700 μ/ml. Membrane preparation were incubated with [^{3}H]DAMGO (1 nM) at 25 °C for 60 min. Nonspecific binding was determined in the presence of 10 μM DAMGO.

2.6. [^{3}S]GTPγS binding assay to recombinant human μ-opioid receptor

CHO cells expressing human μ receptor (PerkinElmer Life Sciences, Boston, MA) were pelleted and suspended in the assay buffer (20 mM HEPES buffer containing 100 mM NaCl, 10 mM $MgCl_2$, 0.2% bovine serum albumin, 3 μW M GDP and 10 μg/ml saponin (pH 7.4)). Membranes were pre-incubated with various concentrations of test compounds for 20 min at 30 °C. [^{35}S]GTPγS (0.15 nM) was then added, and incubated for 60 min at 30 °C. The reaction was terminated by rapid filtration under a vacuum through a UniFilter GF/C microplate (PerkinElmer Life Sciences, Boston, MA) presoaked with assay buffer, after which the filters were washed three times with 0.3 ml of wash buffer (20 mM HEPES buffer (pH 7.4)) using a UniFliter96 harvester (Packard Instruments, Meriden, CT). Microscinti 0 scintillator (Packard Instruments, Meriden, CT) was added, and filter-bound activity was counted in a TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J (Packard Instruments, Meriden, CT). Nonspecific binding was determined in the presence of 10 μM GTPγS.

2.7. Carrageenan-induced mechanical hyperalgesia in rats

The ability of the compound to reverse carrageenan-induced hyperalgesia was determined using the method described by Boyce et al. (1994). The paw pressure threshold to a noxious mechanical stimulus was determined using a modified Ugo Basile algesiometer. The animal's hind paw was positioned over a convex surface (radius 2.5 mm) and gradually increasing pressure was applied to the frontal surface until the paw was withdrawn. Rats were then received an intraplantar (i.pl.) injection of carrageenan (1.5 mg in 0.15 ml) or saline (0.15 ml) into one hind paw, and mechanical threshold of the hind paw was redetermined 3 h later. Carrageenan-induced hyperalgesia was defined as the difference in withdrawal threshold between rats that had been received the injection of saline and those receiving carrageenan. HON0001 (p.o.) or morphine (s.c.) was administered 2.5 h after injection of carrageenan. The measurement was conducted 30 min after HON0001 or morphine injection.

2.8. Hyperalgesia in rats following partial sciatic nerve ligation

The ability of HON0001 to alter the mechanical hyperalgesia accompanying with nerve injury was tested in rats as described by Seltzer et al. (1990) with modification. The right sciatic nerve was exposed at high-thigh level. In sham-operated rats, the nerve was left intact and the wound was closed with 3–0 silk suture. In experimental animals, the dorsum of the nerve was carefully freed from surrounding connective tissue. A 3–0 silk suture was inserted into the nerve and tightly ligated so that the dorsal 1/3–1/2 of the nerve thickness was trapped in the ligature. The wound was closed as in sham-operated rats. Two weeks after the operation, mechanical sensitivity to touch was measured with a set of von Frey filaments with incremental bending forces from 0.16 to 26.0 g. The rat was placed in a chamber with mesh metal floor and walls. The box enables the rat to walk freely but not to rear up on its hind limbs. The experimenter was able to reach the plantar surface of the paws from beneath so that rat was unable to observe the von Frey hair. HON0001 was injected orally 60 min prior to the test. Each hair was indented in the mid-plantar skin until it just bent. The test was repeated 10 times until the rat flipped the paw quickly. If no flipping was observed, the hair was changed to a harder one.

2.9. Spontaneous locomotor activity in rats

Rats were housed individually in transparent acrylic cages (47×28.5×29.5 cm) in a SCANET (Neuroscience Inc., Tokyo,

Table 1
Effect of HON0001 and ifenprodil on [^{3}H]MK-801 and [^{3}H]ifenprodil binding

Compound	[^{3}H]MK-801 binding		[^{3}H]Ifenprodil binding
	High affinity (NR2B sites)	Low affinity (NR2A sites)	
		IC_{50} (nM)	
Ifenprodil	49.59±16.31	22,130±5800	22.41±2.70
HON0001	54.68±4.96	46,480±5850	57.01±3.40

Data represents mean±S.E. obtained from 3 to 4 separate experiments, each done in duplicate.

Japan) placed in a sound-proof box, and acclimatized for 60 min. Immediately after administration of MK-801 (i.p.) or HON0001 (p.o.), locomotor activity was recorded every 10 min for 120 min.

2.10. Rotarod performance in rats

The rotarod (Campdem Instruments, Leicestershire, UK) consisted of a gritted plastic roller (3 cm diameter, 9 cm long) flanked by two large round plates to prevent the animal from escaping and was run at 10 rpm. All rats were given control trials before the test. A rat was placed on the roller, and the length of time it remained on the rod was measured. A maximum of 2 min was allowed for each animal. MK-801 (i.p.) or HON0001 (p.o.) was administered 60 min prior to the test.

2.11. Passive avoidance test in rats

The apparatus consisted of two compartments: one light compartment (12 cm long, 12 cm wide and 25 cm high) and one dark compartment (30 cm long, 12 cm wide and 25 cm high), connected by a guillotine door. Rats were allowed to stay in the light compartment for 1 min, and then the guillotine door was opened. Once the rat had entered the dark compartment, the guillotine door was closed, and rats were allowed to stay in the dark compartment for 2 s, then an electrical shock (2.5 mA for 3 s) was delivered to the animal via the metal grid bars of the floor (acquisition trial). After being allowed to stay in the dark compartment for 10 s, the animal was returned to its home cage until the retention trial. The retention trial was carried out 24 h after the acquisition trial. At that time, the animals were returned to the light compartment, and allowed to stay in the light compartment for 1 min, and the guillotine door was opened. The time taken to enter the dark compartment (step-through latency: STL) was recorded. A maximum latency of 300 s was used. MK-801 (i.p.) or HON0001 (p.o.) was administered 60 min prior to the acquisition trial.

2.12. Measurement of brain-to-plasma ratios

HON0001 was administrated intravenously at 1 mg/kg. After taking a blood sample from the tail vein, rats were decapitated at 1 or 6 h after administration. The brain was immediately excised and weighed. A 4-fold volume of water was added to each tissue, which was then homogenized. All samples were stored at −80 °C until analyzed. To a 50-μl aliquot of plasma or brain sample, 200 μl of imipramine (250 ng/ml) working CH_3CN solution was added, and the tube was vortexed and centrifuged at 2000×*g* at 4 °C for 10 min. The 5-μl supernatant was injected into the LC/ESI-MS/MS system. Chromatographic separation was achieved on a Hypersil BDS-C18 (5 mm, 50 mm×4.6 mm I.D.) with a linearly increasing gradient of 5–95% acetonitrile containing 0.1% acetic acid against 0.1% acetic acid for 4.5 min at a flow rate of 0.2 ml/min. Tandem mass spectrometric detection was carried out using turbo ionspray in positive ESI ion mode for the parent compound HON0001 (*m/z* 366→176) and imipramine (*m/z* 281→86). The lower limits of quantitation (LLOQ) of plasma and brain were 3 ng/ml and 5 ng/g, respectively. Plasma and brain concentrations were expressed as free form of HON0001.

2.13. Data analysis

The concentration of the test compound that caused 50% inhibition of specific binding (IC_{50} value) was determined from each concentration–response curve. IC_{50} values were determined by the Marquardt–Levenberg nonlinear least-squares curve-fitting procedure, using the MicroCal ORIGIN program (MicroCal, Northampton, MA). Data from in vivo experiments were analyzed by one-way ANOVA and significant differences

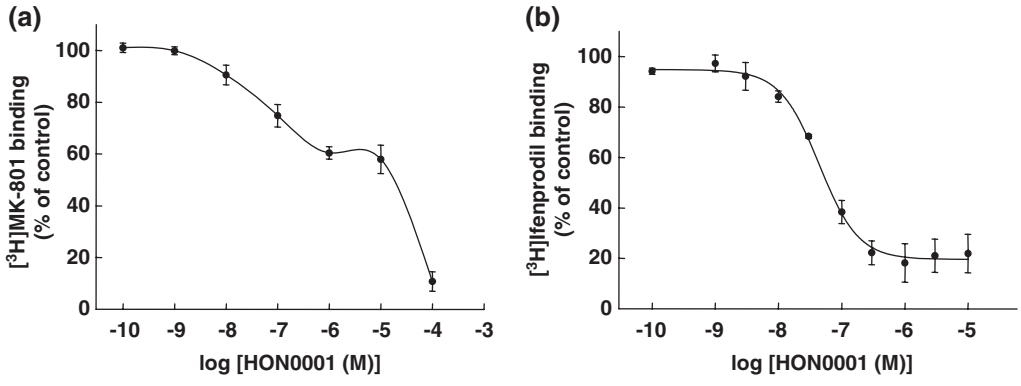

Fig. 2. Displacement of [^{3}H]MK-801 binding to brain membranes (a) and [^{3}H]ifenprodil binding to cerebral cortical membranes (b) by HON0001. Each receptor binding assay was performed as described in Materials and methods. Results are mean values of three to four separate experiments, each done in duplicate.

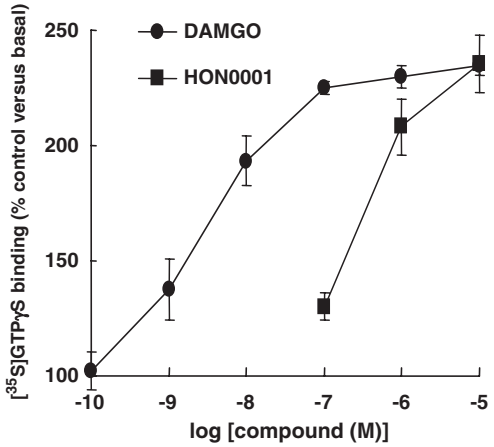

Fig. 3. Effect of HON0001 on [^{35}S]GTPγS binding to human recombinant μ-opioid receptor. [^{35}S]GTPγS binding assay was performed as described in Materials and methods. Data are expressed as % of basal [^{35}S]GTPγS binding obtained from three experiments, each done in duplicate.

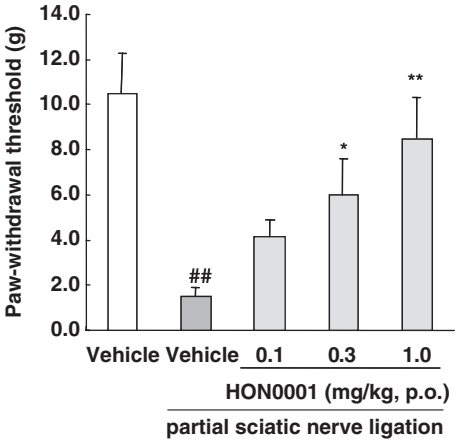

Fig. 5. Reversal by HON0001 of mechanical hyperalgesia resulting from partial sciatic nerve ligation in rats. The right sciatic nerve was partially ligated. Two weeks after surgery, rats were orally administered vehicle or HON0001 60 min prior to the test, and mechanical sensitivity to tactile stimuli was measured with von Frey hairs. Results are expressed as the threshold force required to elicit a response (g) and are the mean±S.E. of 8 rats. $^{\#\#}p<0.01$ versus sham-operated rats; $*p<0.05$, $**p<0.01$ versus vehicle (nerve-ligated rats) (Dunnett's test).

between groups were determined using Dunnett's test. For the passive avoidance task, between-group comparisons were preformed using the Steel test.

3. Results

3.1. In vitro profile of HON0001

Ifenprodil, an NR2B antagonist, inhibited [^{3}H]MK-801 binding in a biphasic manner with IC_{50} values of 49.59±16.31 nM and 22.13±5.8 μM for high- and low-affinity sites, respectively (Table 1). HON0001 inhibited [^{3}H]MK-801 binding with IC_{50} values of 54.68±4.96 nM and 46.48±5.85 μM for high- and low-affinity sites, respectively (Fig. 2a, Table 1). HON0001 inhibited [^{3}H]ifenprodil binding to rat cerebral cortical membranes with an IC_{50} value of 57.01±3.4 nM (Fig. 2b, Table 1), consistent with the data for high-affinity sites of [^{3}H]MK-801 binding. HON0001 exhibited moderate affinity for μ-opioid receptors, with an IC_{50} value of 595 nM, and inhibited dopamine D_1 receptor binding by 79% at 1 μM, as determined by [^{3}H]SCH23390 binding to human recombinant D_1 receptor performed at Cerep. HON0001 did not show an apparent affinity for 40 other receptors, transporters and ion channels, including adrenergic and serotonergic receptors and glutamate recognition sites (data not shown). HON0001 concentration-dependently increased [^{35}S]GTPγS binding to recombinant human μ-opioid receptors to the same extent as DAMGO did, indicating that HON0001 acts as a full agonist at μ-opioid receptors (Fig. 3).

3.2. Antinociceptive activity of HON0001 in rats

In control rats, carrageenan decreased the nociceptive threshold of mechanical hyperalgesia (Fig. 4a,b). HON0001

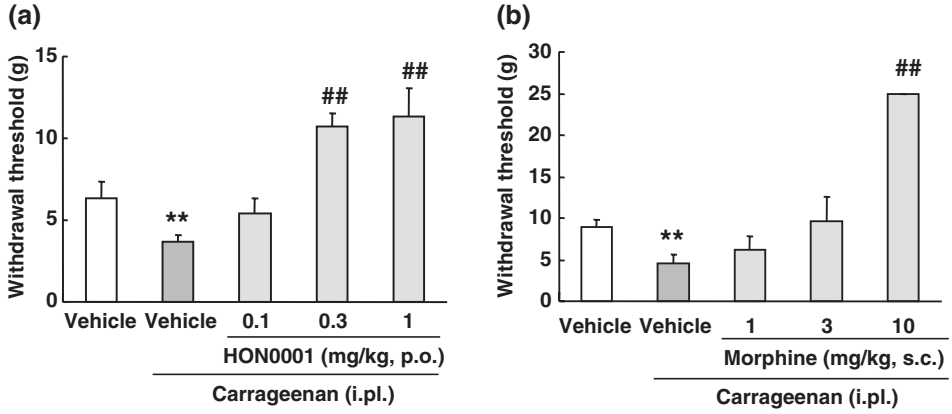

Fig. 4. Reversal by HON0001 and morphine of carrageenan-induced hyperalgesia in rats. Rats were received intraplantar injection of carrageenan into one hind paw, and mechanical threshold of the hind paw was determined 3 h later using a modified Ugo Basile algesiometer. HON0001 was administered 30 min prior to measure withdrawal threshold. Results are expressed as the mean±S.E. of 8 rats. $**p<0.01$ versus carrageenan non-treated group; $^{\#\#}p<0.01$ versus vehicle (carrageenan-treated group) (Dunnett's test).

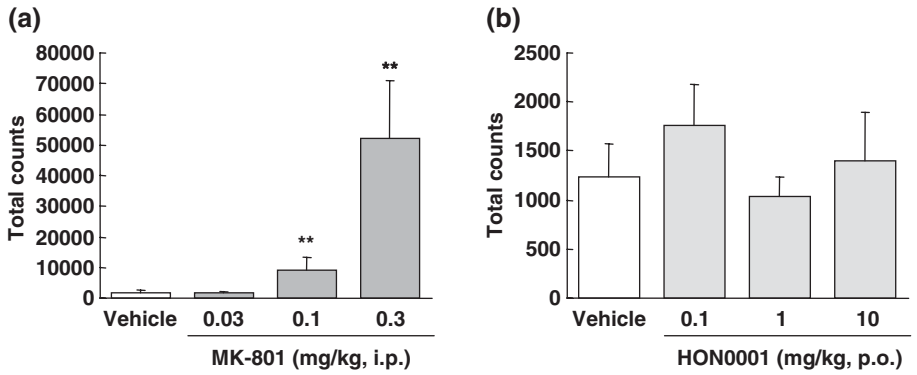

Fig. 6. Effects of HON0001 and MK-801 on spontaneous locomotor activity in rats. Immediately after administration of HON0001 (p.o.) or MK-801 (i.p.), locomotor activity was measured for 120 min. Results are expressed as the mean±S.E. of 6 rats. $**p<0.01$ versus vehicle (Dunnett's test).

(0.1–1 mg/kg), when administered orally, dose-dependently and significantly reversed the decrease in paw withdrawal threshold induced by carrageenan (Fig. 4a). Likewise, subcutaneous administration of morphine, at a dose of 10 mg/kg, significantly inhibited the decrease in nociceptive threshold (Fig. 4b).

HON0001 was tested for its ability to reverse mechanical allodynia behavior in the partial sciatic nerve ligation model of neuropathic pain, in which potent tactile hyperalgesia is induced 14 days postsurgery (Fig. 5). Oral administration of HON0001 dose-dependently and significantly reversed mechanical allodynia behavior in this model of neuropathic pain following oral administration at 0.3 and 1.0 mg/kg (Fig. 5).

3.3. CNS side effect liabilities

Intraperitoneal administration of MK-801 markedly stimulated locomotor activity in rats (Fig. 6a). In contrast, oral administration of HON0001 had no effect on spontaneous locomotor activity of habituated rats even at 10 mg/kg (Fig. 6b). HON0001 did not impair rotarod performance, even at 10 mg/kg, p.o. (Fig. 7b), while MK-801 potently and significantly impaired it (Fig. 7a). Oral administration of HON0001 did not affect step-through latency at even 10 mg/kg. In contrast, MK-801 significantly reduced it at 0.3 mg/kg, i.p. (Table 2).

3.4. Brain penetration

Brain levels of HON0001 were determined at 1 and 6 h after intravenous administration. Following intravenous administration of HON0001 at 1 mg/kg, a high concentration of HON0001 was detected in the brain at 1 h. The mean brain concentration was 827.4 ng/g and the mean plasma concentration 24.0 ng/g, making the mean brain-to-plasma ratio 34.5. Although the mean brain level of HON0001 at 6 h was 25.0 ng/g, HON0001 was not detected in plasma 6 h after intravenous dosing.

4. Discussion

The present study demonstrated that HON0001 is an NR2B antagonist with antinociceptive activity in both inflammatory and neuropathic pain models following oral administration. Furthermore, HON0001, unlike MK-801, was devoid of CNS side effects such as impairment of motor coordination and cognition, and psychotomimetic effects.

HON0001 inhibited [^{3}H]MK-801 binding in a biphasic manner, as did ifenprodil, an NR2B antagonist. It was reported

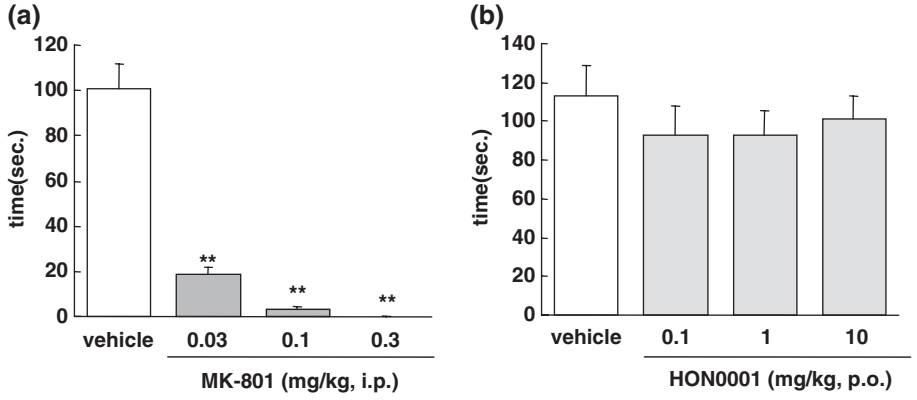

Fig. 7. Effects of HON0001 and MK-801 on rotarod performance in rats. HON0001 (p.o.) or MK-801 (i.p.) was administered 60 min prior to the test. Results are expressed as the means±S.E. of 6 rats. $**p<0.01$ versus vehicle (Dunnett's test).

Table 2
Effect of HON0001 and MK-801 on step-through latency in a passive avoidance task in rats

Drugs	mg/kg		N	Step-through latency (s)	
				Median	(Q1–Q3)
Water for injection	–	i.p.	10	300.0	(300.0–300.0)
MK-801	0.03	i.p.	10	239.0	(99.0–300.0)
	0.1	i.p.	10	300.0	(115.0–300.0)
	0.3	i.p.	10	33.0*	(5.0–52.0)
Saline	–	p.o.	10	300.0	(264.0–300.0)
HON0001	0.1	p.o.	10	260.0	(86.0–300.0)
	1	p.o.	10	300.0	(263.0–300.0)
	10	p.o.	10	114.5	(26.0–300.0)

Data represent median, Q1 and Q3 values of 10 rats. MK-801 and HON0001 were administered intraperitoneally and orally 60 min prior to the acquisition trial. Step-through latency was measured with 300 s cutoff time, 24 h after the acquisition trial.
Q1: 25 percentile, Q3: 75 percentile.
* $p<0.01$, significantly different from control group.

that inhibition of [^{3}H]MK-801 binding to high-affinity sites by NR2B selective antagonists was consistent with that of binding to recombinant NR1/NR2B complex, while the inhibitory effect at low-affinity sites was consistent with that at NR1/NR2A (Fischer et al., 1997). Thus, high-affinity sites of [^{3}H]MK-801 binding to rat brain membranes reflect the proportion of NR2B subunit-containing receptors, and HON0001 exhibited selectivity for NR2B receptors. The high affinity of HON0001 for NR2B receptors was confirmed by the finding that HON0001 also inhibited [^{3}H]ifenprodil binding to NR2B receptors with practically the same potency as that observed at high-affinity sites of [^{3}H]MK-801 binding. It has been reported that ifenprodil has affinity for Ca^{2+} channels and adrenergic, histamine and serotonin receptors, and binds with nanomolar affinity to σ binding sites as well (Coughenour and Barr, 2001; Hashimoto and London, 1993). In the present study, HON0001 did not exhibit an apparent affinity for Ca^{2+} channels, σ binding sites and monoamine receptors including α1 adrenoceptors. Thus, HON0001 has better receptor selectivity than ifenprodil.

In the present study, mechanical allodynia in partially nerve-ligated rats was significantly reversed by HON0001 with a lowest active dose of 0.3 mg/kg when HON0001 was administered orally, indicating that HON0001 is a potent and orally active analgesic and that it might be effective for neuropathic pain. This result is consistent with the previous findings that NR2B antagonists such as CP101,606 and Ro 25-6981 were active against the mechanical allodynia induced by chronic nerve ligation injury (Boyce et al., 1999), although HON0001 exerted analgesic activity at a lower dose than these compounds when administered orally. HON0001 exhibited good blood–brain barrier penetration, with a brain-to-plasma ratio of 34.5. This may explain the potent in vivo oral activity of this compound. Notably, this is the first evidence that an NR2B antagonist is effective in the Seltzer model of neuropathic pain.

The antinociceptive effect of HON0001 was also examined in rat carrageenan-induced hyperalgesia, a model of inflammatory pain. HON0001 was as effective in the carrageenan-induced mechanical hyperalgesia in rats as in the nerve ligation model when administered orally, as also observed with other NR2B antagonists (Boyce et al., 1999). This finding suggests that HON0001 may also have a clinical utility for painful conditions other than those caused by nerve damage.

The clinical development of nonselective NMDA receptor antagonists has been encumbered by their small therapeutic index, since they have unacceptable CNS side effects including motor dysfunction, psychotomimetic effects and induction of cognitive deficits (Balster and Willetts, 1996; Boyce et al., 1999). In the present study, MK-801 markedly stimulated locomotor activity, impaired rotarod performance and reduced step-through latency in a passive avoidance task at 0.3 mg/kg, the dose at which it was reported to exhibit antinociceptive activity (Boyce et al., 1999). Moreover, ifenprodil causes behavioral toxicity, which was evident as a reduction in spontaneous activity (Boyce et al., 1999). In contrast, a significant dose window was observed for HON0001 in the present study. HON0001 failed to affect locomotor activity and rotarod performance at even 10 mg/kg, more than 30 times the dosage exerting antinociceptive activity. These results clearly show that the dose window of HON0001 between antinociceptive effects and motor side effects is much larger than those of both nonselective NMDA receptor antagonists and ifenprodil.

HON0001 did not disrupt cognition in a passive avoidance test, although the analgesic activity of HON0001 may possibly affect the results of this test. Given the report that mice overexpressing the NR2B subunit exhibited improved recognition memory (Tang et al., 2001), the effect of HON0001 on cognition requires assessment in other models, particularly with long-term treatment. However, it has been reported that CP-101,606 did not affect acquisition in the Morris water maze (Guscott et al., 2003), suggesting that NR2B subunit blockade may not have a large impact on spatial memory.

In the present study, we found that, in addition to NR2B receptors, HON0001 exhibited moderate affinity for μ-opioid receptors, and that it acted as a full agonist, as determined by [^{35}S]GTPγS binding, which may significantly affect the outcomes of antinociceptive studies. However, pharmacokinetic study revealed that brain concentration of HON0001 may not be sufficient for agonistic effects at μ-opioid receptors at antinociceptive doses (1 and 3 mg/kg, p.o.), and HON0001 did not cause behavioral abnormalities including sedation observed in the treatment with morphine even at 10 mg/kg, p.o. Therefore, HON0001 may not display μ-opioid receptor agonism in vivo following oral administration, and the analgesic effects of HON0001 following oral administration may be ascribed to the blockade of NR2B receptors. However, because difference in affinity between NR2B receptor and μ-opioid receptor is within one order, involvement of μ-opioid receptor agonistic activity in the effects of HON0001 cannot be fully ruled out.

In conclusion, HON0001 has potent oral analgesic activity in animal models of inflammatory and neuropathic pain at doses

causing no behavioral abnormalities, which are noted with treatment with currently available nonselective NMDA receptor antagonists.

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