Pharmacological Profiles of the Novel Analgesic M58996 in Rat Models of Persistent and Neuropathic Pain

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Abstract. We investigated the effects of 4-(N-{1-[2-(4-cyanophenyl)ethyl]-4-hydroxy-piperidin-4-ylmethyl}-N-methylamino)benzoic acid monohydrochloride (M58996), a novel analgesic, on persistent and neuropathic pain in rats. In the formalin test, oral M58996 (0.3 – 10 mg/kg) reduced nociceptive behaviors only in the late phase. In the neuropathic pain model, oral M58996 (1 – 10 mg/kg) attenuated mechanical allodynia and heat hyperalgesia in the nerve-injured paw without affecting normal responses of the uninjured paw. High doses (10 – 100 mg/kg) of oral M58996 did not influence normal motor function. Thus, M58996 had a wide dose range showing antinociceptive, antiallodynic, and antihyperalgesic effects without motor dysfunction. In addition, we studied the possible mechanisms involved in the M58996-induced antinociception. The antinociceptive effect of M58996 was reversed by intrathecal pertussis toxin, an inhibitor of the inhibitory- and other-GTP-binding protein (Gip/o protein), but not by subcutaneous naloxone, an opioid-receptor antagonist. This effect was also reversed by intracerebroventricular or intrathecal tropisetron, a 5-hydroxytryptamine3 (5-HT3)-receptor antagonist, and intraperitoneal bicuculline, a γ-aminobutyric acid A (GABAa)-receptor antagonist. These results suggest that M58996 produces its antinociceptive effect by a pertussis toxin-sensitive G protein mechanism. In addition, the GABA released by the activation of supraspinal and/or spinal 5-HT3 receptors is likely to contribute to the M58996-induced antinociception.

Keywords: neuropathic pain, allodynia, formalin test, 5-HT3 receptor, GABAa receptor

Introduction

Neuropathic pain is a form of chronic pain that arises from functional changes in the pain sensory system after peripheral nerve injury. This pain syndrome cannot be controlled with conventional analgesics, such as opioids and nonsteroidal anti-inflammatory drugs (1). These drugs possess limited efficacy and unacceptable side effects. Therefore, considerable efforts have recently been spent to discover novel analgesic agents with increased efficacy and improved side effect profiles.

We have previously reported that a putative Na+ channel blocker, 4-[2-(4-hydroxy-4-{{N(N-isopropoxy-phenyl)}-{N'-methylamino}methyl}piperidin-1-yl)ethyl]benzonitrile monohydrochloride (M58373), attenuated mechanical allodynia and heat hyperalgesia in chronic constrictive injury rats (2). However, high doses of this compound produced motor dysfunction and tremor, which were considered to be side effects on the central nervous system. To find a novel analgesic agent with fewer side effects, we furthermore synthesized several derivatives of this compound and screened these derivatives in terms of efficacy and central side effects. Consequently, we identified a candidate, 4-(N-{1-[2-(4-cyanophenyl)ethyl]-4-hydroxypiperidin-4-ylmethyl}-N-methylamino)benzoic acid monohydrochloride (M58996) (Fig. 1). However, M58996 had no action on the Na+ channels, unlike the prototype M58373.

In this study, we showed the efficacy of M58996 in...
the formalin test and the neuropathic pain model. We also investigated the influence of this compound at high doses on motor function. In addition, to elucidate the possible mechanisms of M58996, we examined the involvement of opioid receptors, GTP-binding protein (G protein), monoamine- and γ-aminobutyric acid (GABA)-receptor subtypes in its antinociception using their inhibitors.

Materials and Methods

Animals

This study was conducted according to the ethical guidelines of the International Association for the Study of Pain (3). In addition, all experimental procedures mentioned below were approved by the Institutional Animal Use Committee of our laboratory. Male Wistar rats (Japan SLC, Inc., Hamamatsu) and Wistar Hannover rats (Charles River Laboratories Japan, Inc., Yokohama) were used at the age of 5 – 8 weeks. The rats were kept in an air-conditioned and pathogen-free room with temperature of 23 ± 2°C and humidity of 55 ± 10% on a regulated 12-h light/dark cycle. They had free access to standard laboratory chow (CE-2; Clea Japan, Inc., Tokyo) and drinking water. When the compound was given orally in the pain tests, the rats were fasted overnight with free access to drinking water.

Compounds

M58996 and gabapentin were prepared in our laboratory. The following compounds were tested: naloxone methiodide and pertussis toxin (Sigma Chemical Co., St. Louis, MO, USA). The following monoamine- and GABA-receptor antagonists were used: prazosin hydrochloride (Wako Chemicals, Osaka); yohimbine, pindolol, ketanserin tartrate, tropisetron, (−)-baclofen and picrotoxin (Sigma). The doses of these compounds were estimated in the preliminary experiments or according to previous studies (4 – 6). In all experiments, an equal volume of the vehicle was used as the control.

M58996 and gabapentin were dissolved in 0.5 w/v% hydroxypropylmethylcellulose. Pertussis toxin was dissolved in phosphate-buffered saline that contained 1 mg/ml bovine serum albumin. Prazosin hydrochloride, yohimbine, and ketanserin tartrate were dissolved in 10% dimethylsulfoxide and 90% water. Pindolol was dissolved in 1.2 mM HCl. Other compounds were dissolved in physiological saline.

The rat formalin test

The experiment was conducted according to our previous report (6). The rats were initially acclimated to the acryl cages (Muromachi Kikai, Tokyo) for 15 min before the formalin injection. Formalin (50 µl of 0.5% formaldehyde solution in saline) was subcutaneously injected into the plantar surface of the rat’s left hind paw. Nociceptive behaviors were quantified by measuring the time spent in licking/biting the injected paw every 5 min with a stopwatch. Changes in the time spent in nociceptive behaviors was biphasic. The recording of the early phase started immediately after the formalin injection and lasted for 10 min; the recording of the late phase started after the early phase and lasted for 35 min. The total time spent engaging in these behaviors was calculated in each phase. At the end of the experiment, the formalin-injected rats were killed by the inhalation of CO2 gas.

M58996 and gabapentin were given orally (10 ml/kg) 30 min before the formalin injection (that is, about 1 h before the peak of late phase). Pertussis toxin was pretreated intrathecally (10 µl/site) 1 day before the M58996 treatment (7). Naloxone methiodide was pretreated subcutaneously (2 ml/kg). The a-adrenoceptor antagonists (prazosin hydrochloride and yohimbine) and 5-hydroxytryptamine (5-HT)-receptor antagonists (pindolol, ketanserin tartrate, and tropisetron) were pretreated intracerebroventricularly (5 µl/site) or intrathecally. The GABA-receptor antagonists (bicuculline methiodide and saclofen) were pretreated intraperitoneally (2 ml/kg). In addition, the subcutaneous and intraperitoneal pretreatments of the antagonists were done 15 min before the M58996 treatment. The intracerebroventricular and intrathecal pretreatments of the antagonists except pertussis toxin were done 5 min before the M58996 treatment.

The percent reversal by an antagonist in the M58996-induced antinociception was calculated according to the following formula:

Percent reversal (%) = \( 1 - (NB_{vehicle} - NB_{antagonist/M58996}) / (NB_{vehicle} - NB_{M58996}) \) \times 100

\( NB_{vehicle} \): nociceptive behaviors in the vehicle-treated group, \( NB_{antagonist/M58996} \): nociceptive behaviors in the antagonist/M58996-treated group, \( NB_{M58996} \): nociceptive behaviors in the M58996-treated group.
Chronic constrictive injury model of neuropathic pain

The chronic constrictive injury model was produced according to the method of Bennett and Xie (8). Wistar Hannover rats were anesthetized by intraperitoneal injection with sodium pentobarbital (45 mg/kg). The left common sciatic nerve was exposed by blunt dissection, and 4 loose ligatures (3 – 0 chromic gut; Matsuda Ika Kogyo Co., Ltd., Tokyo) were placed around the nerve at intervals of 1 mm. Then, the muscle and skin were sutured. As a sham operation, the right sciatic nerve was isolated in the same way, but it was not ligated. After the operation, to minimize discomfort and painful mechanical stimulation, the rats were housed in the plastic cages with soft bedding. The rats at 14 – 15 days after the operation were used for the following behavioral tests. At this time, about 80% of these rats developed distinct neuropathic behaviors such as allodynia and hyperalgesia.

Behavioral tests

Mechanical allodynia was assessed according to the method of Seltzer et al. (9). The neuropathic rats were individually placed in the plastic cages with mesh bottoms. The withdrawal thresholds to mechanical stimuli were measured with a set of von Frey filaments (Stoelting Company, Wood Dale, IL, USA) ranging from 0.69 to 28.84 g. Each filament was vertically applied to the mid-plantar skin in ascending order in a period of 3 s. At the thresholds, the rats responded with a quick paw withdrawal. When no response was observed, the force of the thickest filament (28.84 g) was assigned as the withdrawal threshold.

Heat hyperalgesia was assessed according to the method of Hargreaves et al. (10). The neuropathic rats were individually placed in transparent plastic chambers with a glass floor and acclimated to them for 15 min. The radiant heat source (Plantar test No. 7370; Ugo Basile, Comerio, VA, Italy) was aimed through the glass onto the mid-plantar area of both paws. The intensity of heat stimuli was kept constant throughout the experiment. Each paw was tested 2 times, at an interval of at least 3 min. The latency from initial heat activation to paw withdrawal was recorded to the nearest 0.1 s as the withdrawal latency. A cut-off latency of 30 s was used to avoid tissue damage.

In these tests, the rats were orally treated with one dose of M58996 or gabapentin only once and tested in a blinded manner.

Motor function

Locomotor activity and motor coordination were assessed with Supermex (Muromachi Kikai) and accelerating rotarod apparatus (model 7750, Ugo Basile), respectively. On the first day, locomotor activities of the rats were measured, and then the rats were trained twice on the rotarod. They were allocated to each group so that there would not be any considerable difference among the groups in terms of these mean values. On the second day, the rats were orally treated with M58996 and gabapentin; and then 60 min later, they were individually placed into the acryl cages and locomotor activity was recorded for 10 min. At 90 min after the treatment, they were placed onto the accelerating rotarod which increased in speed from 4 to 40 rpm over 5 min, and then the time required for the rat to fall from the rod was recorded with a maximum cut-off of 300 s.

Statistical analyses

Results are expressed as means ± S.E.M. Statistical analysis among multiple groups was performed by one-way analysis of variance followed by Dunnett’s post hoc test. In the formalin test with various inhibitors, comparisons between two groups were made by Student’s t-test. For all the analyses, P values less than 0.05 were considered significant.

Results

Effects of M58996 in the rat formalin test

Figure 2 shows the effects of oral M58996 (0.3 – 10 mg/kg) and gabapentin (10 – 100 mg/kg) in the rat formalin test. Both M58996 and gabapentin showed no effect on the time spent in nociceptive behaviors in the early phase, but they reduced it in the late phase in a dose-dependent manner. Statistically significant effects in the late phase were observed at 1 mg/kg or higher of M58996 (Fig. 2A) and at 30 and 100 mg/kg of gabapentin (Fig. 2B).

Effects of M58996 on mechanical allodynia

Figure 3 shows the effects of oral M58996 (1 – 10 mg/kg) and gabapentin (10 – 100 mg/kg) on mechanical allodynia in the chronic constrictive injury rats. In the vehicle-treated group, the withdrawal thresholds of the nerve-injured and uninjured paws were 2.2 ± 0.3 and 14.1 ± 0.5 g, respectively. The decrease in the withdrawal threshold of the nerve-injured paw indicates mechanical allodynia. M58996 attenuated this mechanical allodynia in a dose-dependent manner. Statistically significant effects were observed at 3 and 10 mg/kg (Fig. 3A). In the uninjured paw, M58996 showed no effect (100% – 103% of the pre-value). On the other hand, gabapentin also attenuated this mechanical allodynia in a dose-dependent manner. Statistically significant effects were observed at 30 and 100 mg/kg
However, gabapentin at 100 mg/kg tended to increase the normal withdrawal threshold of the uninjured paw (up to 163% of the pre-value, $P = 0.055$).

Effects of M58996 on heat hyperalgesia

Figure 4 shows the effects of oral M58996 (1 – 10 mg/kg) and gabapentin (10 – 100 mg/kg) on heat hyperalgesia in the chronic constrictive injury rats. In the vehicle-treated group, the withdrawal latencies of the nerve-injured and uninjured paws were 9.7 ± 0.7 and 15.1 ± 0.7 s, respectively. The decrease in the withdrawal latency of the nerve-injured paw indicates heat hyperalgesia. M58996 attenuated this heat hyperalgesia in a dose-dependent manner. Statistically significant effects were observed at 3 and 10 mg/kg (Fig. 4A). M58996 showed no effect (98% – 109% of the pre-value) on the uninjured paw. On the other hand, gabapentin at 100 mg/kg significantly attenuated this heat hyperalgesia (Fig. 4B), without affecting the normal withdrawal threshold of the uninjured paw (93% – 105%...
Influence of M58996 on motor function

Figure 5 shows the influence of oral M58996 (10 – 100 mg/kg) and gabapentin (300 mg/kg) on locomotor activity and motor coordination. M58996 did not influence locomotor activity, but gabapentin tended to reduce it ($P = 0.095$, Fig. 5A). In addition, M58996 did not influence time spent on the rod, but gabapentin significantly reduced it (Fig. 5B). Thus, even high doses of M58996 did not influence normal motor function, unlike gabapentin.

Involvement of opioid receptors and $G_{i/o}$ protein in M58996-induced antinociception

In the rat formalin test, oral M58996 at 10 mg/kg
significantly reduced nociceptive behaviors in the late phase ($P<0.01$). Subcutaneous pretreatment with the opioid-receptor antagonist naloxone methiodide (4 mg /kg) did not reverse the M58996-induced antinociception (3.1% reversal, $n=5$). On the other hand, intrathecal pretreatment with the $G_{i/o}$-protein inhibitor pertussis toxin (1 µg/site) reversed this antinociception (74.5% reversal, $n=5$) (Fig. 6). In addition, these inhibitors alone did not modify the late-phase reaction compared with that of the vehicle-treated group.

Involvement of monoamine- and GABA-receptor subtypes in M58996-induced antinociception

Intracerebroventricular pretreatment with the 5-HT$_3$-receptor antagonist tropisetron (1 ng/site) reversed the antinociceptive effect of oral M58996 (10 mg/kg) on the late-phase reaction in the rat formalin test (83.3% reversal, $n=5$) (Fig. 7A). However, intracerebroventricular pretreatment with the $\alpha_1$-adrenoceptor antagonist prazosin hydrochloride (1 µg/site), $\alpha_2$-adrenoceptor antagonist yohimbine (3 µg/site), 5-HT$_{1A/1B}$-receptor antagonist pindolol (1.5 µg/site), or 5-HT$_2$-receptor antagonist ketanserin tartrate (6 µg/site) did not reverse the M58996-induced antinociception (12.6%, −4.0%, −8.5%, or 27.0% reversal, respectively, $n=5$ for all experiments). Intrathecal pretreatment with tropisetron (10 µg/site) also reversed this antinociception (78.8% reversal, $n=5$). On the other hand, intraperitoneal pretreatment with the GABA$_A$-receptor antagonist bicuculline methiodide (0.5 mg/kg) reversed the M58996-induced antinociception (113.6% reversal, $n=5$) (Fig. 7B), but the GABA$_B$-receptor antagonist saclofen (11.2 mg/kg) did not reverse this antinociception (12.4% reversal, $n=5$). In addition, these monoamine- and GABA-receptor antagonists alone did not modify the late-phase reaction compared with that of the vehicle-treated group.
Discussion

In this study, we synthesized several derivatives of the prototype M58373 (2) and then screened these compounds using the formalin test and general observation test. The formalin test is a valid model for clinical pain because of the moderate and continuous pain generated by tissue injury. The responses to formalin show early and late phases, and the late-phase reaction is considered to be persistent pain associated with the inflammation (11–13). As a result, we found M58996 that reduced the late-phase reaction without inducing abnormal behaviors. On the other hand, the chemical structure of M58996 was strikingly similar to that of M58373, a Na\(^+\) channel blocker (2), but M58996 had no binding affinity (4% inhibition at 10 \(\mu\)M) to the Na\(^+\) channels (14). To clarify the target molecule of M58996, we have conducted 125 biochemical assays (Spectrum-Screen\(^\text{®}\); MDS Pharma Services, Taipei, Taiwan) that include radioligand binding assays for receptors (opioid, adrenergic, 5-HT\(_3\)ergic, GABAergic, etc.); transporters (noradrenaline, 5-HT, GABA, etc.); and channels (Na\(^+\), Ca\(^{2+}\), etc.). However, no binding affinity was observed in these assays even at 10 \(\mu\)M (data not shown). Further studies are required to elucidate the mechanism of action of M58996.

Allodynia (pain response to non-noxious stimuli) and hyperalgesia (increased pain response to noxious stimuli) are taken to be the most prominent symptoms of neuropathic pain syndrome. It has been reported that mechanical allodynia is caused by the sprouting of A\(\delta\) fibers into the superficial laminae in the spinal dorsal horn (15, 16). By contrast, noxious heat stimuli are transmitted to the spinal cord through C- and some A\(\delta\) fibers. Thus, different processes exist in the transmission of mechanical allodynia and heat hyperalgesia. In this study, M58996 attenuated both mechanical allodynia and heat hyperalgesia in the nerve-injured paw without affecting normal responses of the uninjured paw. In addition, even high doses of this compound did not influence normal motor function, unlike gabapentin (17–19). These results suggest that M58996 has a wider dose range showing analgesic effects without affecting normal function than gabapentin.

An additional objective in this study was to evaluate the mechanisms of M58996-induced antinociception by use of various inhibitors. First, we investigated the involvement of the opioidergic system in the M58996-induced antinociception. This antinociception was not reversed by the systemic opioid-receptor antagonist. Considering that M58996 did not bind to opioid receptors such as \(\mu\)-, \(\delta\)-, and \(\kappa\)-receptors, these findings suggest that opioid receptors and endogenous opioid peptides are not involved in the antinociception of M58996. Second, we studied the involvement of G-protein-coupled receptors in the M58996-induced antinociception. This antinociception was reversed by the intrathecal G\(_{i/o}\)-protein inhibitor. Thus, G\(_{i/o}\)-protein-coupled receptors other than opioid receptors would be involved in the antinociception of M58996 at the spinal cord.

Monoamines and GABA are implicated in endogenous pain control systems (20), and the sensation of pain is subject to descending control from higher centers. Therefore, third, we investigated the involvement of monoamine- and GABA-receptor subtypes in the antinociception of M58996. Consequently, the intracerebroventricular or intrathecal 5-HT\(_3\)-receptor antagonist and systemic GABA\(_A\)-receptor antagonist reversed the M58996-induced antinociception. The 5-HT\(_3\) receptors are the ligand-gated cation channels mediating membrane depolarization and neuronal excitation, and they are widely distributed from central to peripheral organs (21). A previous behavioral study has indicated that intrathecal 5-HT\(_3\)-induced antinociception is mediated by 5-HT\(_3\) receptors (22). Morales et al. have also shown that the expression of 5-HT\(_3\) receptors is mainly observed on GABA-containing neurons (23). In addition, previous studies have shown that the activation of spinal 5-HT\(_3\) receptors evokes GABA release, resulting in the analgesic effects (24, 25). Taken together, these results suggest that M58996 evokes GABA release through the indirect activation of supraspinal and/or spinal 5-HT\(_3\) receptors, and the released GABA produces antinociceptive effects via GABA\(_A\) receptors. This suggestion is supported by previous studies that 5-HT\(_3\) and GABA\(_A\) receptors play important roles in nociceptive transmission (25–27). In addition, in the monkeys treated with oral M58996 (40 mg/kg), no abnormal findings such as emesis and sedation were observed, although the activation of 5-HT\(_3\) and GABA\(_A\) receptors is considered to induce emesis and sedation, respectively (28, 29). From the above observations, we have speculated that the main action site of M58996 is the spinal cord.

In summary, M58996 showed antinociceptive, anti-allodynic, and antihyperalgesic effects in persistent and neuropathic pain models without affecting normal motor function. The antinociceptive action of M58996 seems to be produced by a pertussis toxin-sensitive G-protein mechanism. In addition, M58996 would enhance inhibitory GABAergic neurons through the indirect activation of 5-HT\(_3\) receptors.
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References