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Potent analgesic effects of a putative sodium channel blocker M58373 on formalin-induced and neuropathic pain in rats

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Abstract

M58373, 4-[2-(4-hydroxy-4-{[*N*-(4-isopropoxyphenyl)-*N*-methylamino]methyl}piperidin-1-yl)ethyl]benzonitrile monohydrochloride, is a novel compound, which has an inhibitory activity on neurotoxin binding to the site 2 of voltage-gated sodium channels. In this study, we investigated the effects of M58373 on substance P release from sensory neurons in vitro and pain behaviors/responses in rats, compared with mexiletine. M58373 (1–10 μM) inhibited veratridine-induced release of substance P from dorsal root ganglion cells. In the formalin test, oral M58373 (0.3–10 mg/kg) reduced the time spent in nociceptive behaviors only in the late phase. In the neuropathic pain model, oral M58373 (1–10 mg/kg) attenuated mechanical allodynia and heat hyperalgesia in the nerve-injured paw without affecting normal responses in the uninjured paw. In contrast, oral mexiletine (10–100 mg/kg) had a narrow therapeutic dose range in both models because of the adverse effects on the central nervous system. These results suggest that M58373 is a favorable prototype for novel anti-neuropathic pain agents.

Keywords: Neuropathic pain; Sodium channel; Dorsal root ganglion; Substance P; Nerve injury

1. Introduction

Peripheral nerve injury causes neuropathic pain which can be manifested as allodynia (pain response to non-noxious stimulus), hyperalgesia (increased response to noxious stimulus) and spontaneous pain (Woolf and Mannion, 1999). This hyperexcitability observed in neuropathic pain results at least partly from the accumulation of voltage-gated sodium channels at the nerve-injured site (England et al., 1994; Matzner and Devor, 1994). In clinical practice, several local anesthetics, anticonvulsants and antiarrhythmics are currently used to manage neuropathic pain (Tanelian and Brose, 1991; Kalso, 2005). These drugs commonly bind to sodium channels to prevent the influx of sodium ion into cells (Clare et al., 2000). In addition, several antidepressants and neuroleptics used for neuropathic pain have been shown to inhibit cell excitability evoked by an alkaloid, veratridine, which activates the voltage-gated sodium channel (Deffois et al., 1996; Urenjak and Obrenovitch, 1996).

To search for a novel anti-neuropathic pain agent, we carried out veratridine-induced cytotoxicity assay using the mouse neuroblastoma cell line (Neuro-2A). This cytotoxicity is caused by the activation of sodium channels (Hamasaki et al., 1996). Consequently, we found M58373, 4-[2-(4-hydroxy-4-{[*N*-(4-isopropoxyphenyl)-*N*-methylamino]methyl}piperidin-1-yl)ethyl]benzonitrile monohydrochloride (Fig. 1), which potently inhibited the cell death.

In this study, we examined the effects of M58373 and a subtype-nonselective sodium channel blocker, mexiletine, on veratridine-induced release of substance P from rat dorsal root ganglion cells in vitro. We also investigated their effects in the rat formalin test and neuropathic pain model in vivo. The data obtained have suggested that M58373 has a wider therapeutic dose range than mexiletine.

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2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983). In addition, all experimental procedures mentioned below were approved by the Institutional Animal Use Committee of our laboratory.

Young adult male Wistar Hannover rats (Charles River Laboratories Japan, Inc. and Clea Japan, Inc.) at the age of 6–9 weeks were used for the experiments. They 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.) and drinking water. When the compound was given orally, they were fasted overnight with free access to drinking water.

2.2. Compounds

M58373 was synthesized in our laboratory. Mexiletine hydrochloride (racemate) was purchased from Sigma Chemical Co. In vitro experiments, these compounds were dissolved in pure water, and then the solutions were diluted to the final concentration with the medium. In vivo experiments, they were dissolved in 0.5 w/v% hydroxypropylmethylcellulose and given orally in a volume of 10 ml/kg. The following reagents were used: tetrodotoxin (Sankyo Co., Ltd.); collagenase A (Roche Diagnostics); trypsin (Worthington Biochemical Co.); nerve growth factor-7S, veratridine, captopril, phosphoramidon sodium (Sigma Chemical Co.); substance P enzyme immunoassay kit (Cayman Chemical). In all experiments, an equal volume of the vehicle was used as the control.

2.3. Culture of rat dorsal root ganglion cells

Dorsal root ganglion cells were prepared according to the method of Inoue et al. (1999). The rats were decapitated, and the spinal columns were surgically removed. The columns were bilaterally incised under a dissecting microscope, and then the dorsal root ganglia were aseptically removed. The ganglia were digested with 0.125 w/v% collagenase and 0.1 w/v% trypsin in Hanks' balanced salt solution. Enzymatic reaction was terminated by adding 2 ml of Hanks' solution containing 10% heat-inactivated fetal bovine serum. The ganglia were mechanically dissociated by trituration through a Pasteur pipette until tissue fragments were no longer visible. After washing in Hanks' solution, the ganglion cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 100 ng/ml nerve growth factor. The cells were placed in 48-well culture plates coated with poly-L-lysine and maintained in the medium at 37 °C in an atmosphere of 5% CO_2 for 3 days. Our preliminary experiments have shown that the dorsal root ganglion cells are positively immunostained for substance P and sensitive to capsaicin (100–300 nM) exposure.

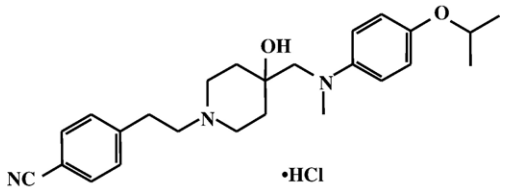

Fig. 1. Chemical structure of M58373.

2.4. Determination of substance P release

The release experiments were performed according to the method of Kessler et al. (1983). The dorsal root ganglion cells were preincubated in Dulbecco's modified Eagle's medium with 25 mM HEPES, 2 mg/ml bovine serum albumin and peptidase inhibitors (10 μM captopril and 10 μM phosphoramidon) containing a test compound at 37 °C for 10 min. After the medium was removed, the cells were incubated in the medium containing 10 μM veratridine and a test compound at 37 °C for 30 min. And then the medium supernatant was collected as the sample for immunoassay.

The substance P content in the supernatant was determined using substance P enzyme immunoassay kit. Optical density at 405 nm was measured with Thermomax microplate reader (Molecular Devices, CA, USA). The concentration of substance P was expressed as picogram per well (pg/well). The limit of detection was 4.1 pg/well. The data presented here are based on 3 independent experiments. Our preliminary experiments have shown that M58373 does not release substance P from these cells by itself.

2.5. Formalin test

The experiment was performed according to our previous report (Akada et al., 2005). The rats were initially acclimated to the acryl cages (Muromachi Kikai, Tokyo, Japan) for 15 min before the formalin injection. Fifty microliters 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 are biphasic. The first reaction between 0 and 10 min after the formalin injection was considered as the early phase, whereas the second reaction between 10 and 45 min after it as the late phase. Compounds were given orally 30 min before the formalin injection (that is, about 1 h before the peak of the late phase). At the end of the experiment, the formalin-injected rats were killed with CO_2 gas.

2.6. Chronic constrictive injury model of neuropathic pain

Chronic constrictive injury model was produced according to the method of Bennett and Xie (1988). The rats were anesthetized with 45 mg/kg, i.p. of sodium pentobarbital. The

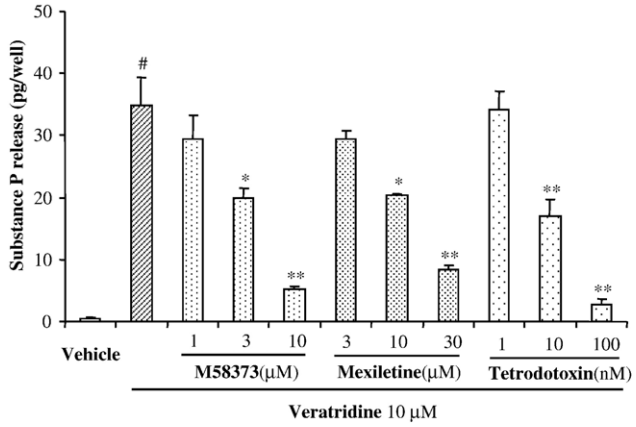

Fig. 2. Inhibitory actions of M58373, mexiletine and tetrodotoxin on veratridine-induced release of substance P from rat dorsal root ganglion cells. Values are expressed as means±S.E.M. of 3 independent experiments. $^{\#}P<0.05$ significantly different from the basal release (Student's *t*-test). $^{*}P<0.05$, $^{**}P<0.01$ significantly different from the group treated with only veratridine (parametric Dunnett's test).

left common sciatic nerve was exposed by blunt dissection at the level of midthigh, and 4 loose ligatures (3–0 chromic gut; Matsuda Ika Kogyo Co., Ltd., Tokyo, Japan) were placed around the nerve. And 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. To minimize discomfort and painful mechanical stimulation, the rats were housed in the plastic cages with floors covered with soft bedding. The neuropathic rats, 14–15 days after the operation, were used for the behavioral testing. At this time, about 80% of the rats developed distinct neuropathic behaviors (allodynia or hyperalgesia).

2.7. Behavioral testing in the neuropathic rats

Mechanical allodynia was assessed according to the method of Seltzer et al. (1990). 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, WI, 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 flick. 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. (1988). 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, 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 latencies from initial heat activation to paw withdrawal were recorded to the nearest 0.1 s as the withdrawal latencies. A cut-off latency of 30 s was used to avoid tissue damage.

In these behavioral tests, the rats were treated with one dose of a compound only once and tested in a blinded manner.

2.8. Motor coordination

Motor coordination was measured with the accelerating rotarod apparatus (model 7750; Ugo Basile, Camerio, Italy). The rats were acclimatized to acceleration by 3 training sessions and assigned to each group based on the values assessed the day before. They were placed onto the rotating rod, which increased in speed from 4 to 40 rpm over 5 min. The time required for the rat to fall from the rod was recorded, with a maximum cut-off of 300 s. M58373 and mexiletine were given orally 1 h before the measurement.

2.9. General observation test

The rats were observed for adverse behaviors such as general changes in activity level, seizure activity and tremor. In the formalin test, the symptoms of rats were observed for 30 min after the treatment with a compound. In neuropathic rats and motor coordination test, those were observed for 1 h after it.

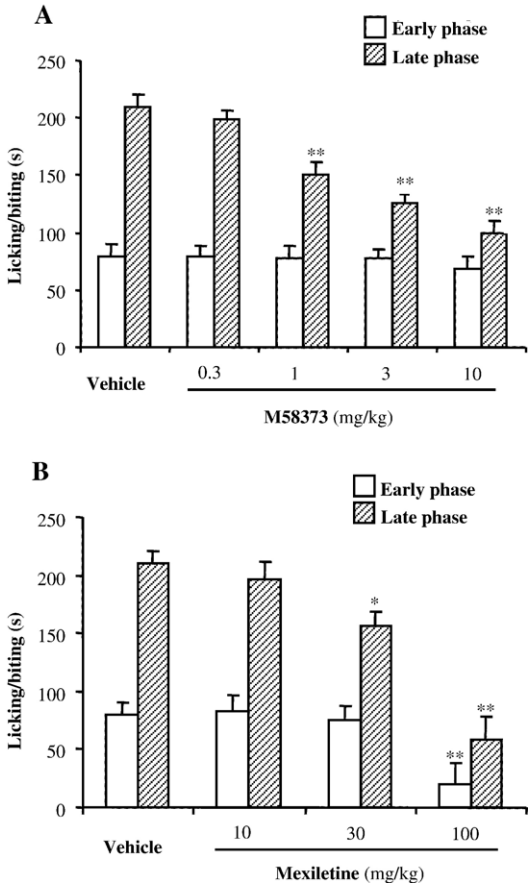

Fig. 3. Effects of M58373 and mexiletine on formalin-induced nociceptive behaviors in the early and late phases in the rat formalin test. Values are expressed as means±S.E.M. of 8 animals. $^{*}P<0.05$, $^{**}P<0.01$ significantly different from the vehicle-treated group (parametric Dunnett's test).

2.10. Statistical analysis

Results were expressed as means ± S.E.M. Statistical analysis among multiple groups was performed with Dunnett's test. Comparison between two groups was made using Student's *t*-test. The allodynia data over time were analyzed by repeated measure analysis of variance on ranks (Friedman test), with Dunn's post hoc test. The hyperalgesia data over time were analyzed by 2-way repeated measure analysis of variance with Dunnett's post hoc test (SAS; SAS Institute, NC, USA). For all the analyses, P values less than 0.05 were considered significant. In addition, concentration–effect data were analyzed for the 50% inhibitory concentration (IC_{50}) values by linear regression using JMP Version 4.0.2 (SAS Institute).

3. Results

3.1. Effects of M58373 on veratridine-induced substance P release

Fig. 2 shows the effects of M58373 (1–10 μM), mexiletine (3–30 μM) and tetrodotoxin (1–100 nM) on veratridine-induced release of substance P from rat dorsal root ganglion cells. Veratridine (10 μM) markedly increased the substance P release. M58373 inhibited the substance P release in a concentration-dependent manner. The sodium channel blockers, mexiletine and tetrodotoxin, also inhibited it in a concentration-dependent manner. The IC_{50} values of M58373, mexiletine and tetrodotoxin were 3.28, 12.0 μM and 10.3 nM, respectively. In addition, these compounds showed no toxic effects on the cells (data not shown).

3.2. Effects of M58373 in the rat formalin test

Fig. 3 shows the effects of oral M58373 (0.3–10 mg/kg) and mexiletine (10–100 mg/kg) in the rat formalin test. M58373 showed no effect on the time spent in nociceptive behaviors in the early phase but reduced it in the late phase in a dose-dependent manner. Statistically significant effects in the late phase were observed at 1 mg/kg and higher (Fig. 3A). In contrast, mexiletine significantly reduced them in the early phase at 100 mg/kg and in the late phase at 30 and 100 mg/kg (Fig. 3B).

Though M58373 showed no adverse effects, mexiletine induced tonic–clonic convulsion and decreased activity level in 2 out of 8 rats at 100 mg/kg.

3.3. Effects of M58373 on mechanical allodynia

Fig. 4 shows the effects of oral M58373 (1–10 mg/kg) and mexiletine (10–100 mg/kg) on the withdrawal thresholds of the nerve-injured and uninjured paws in the chronic constrictive

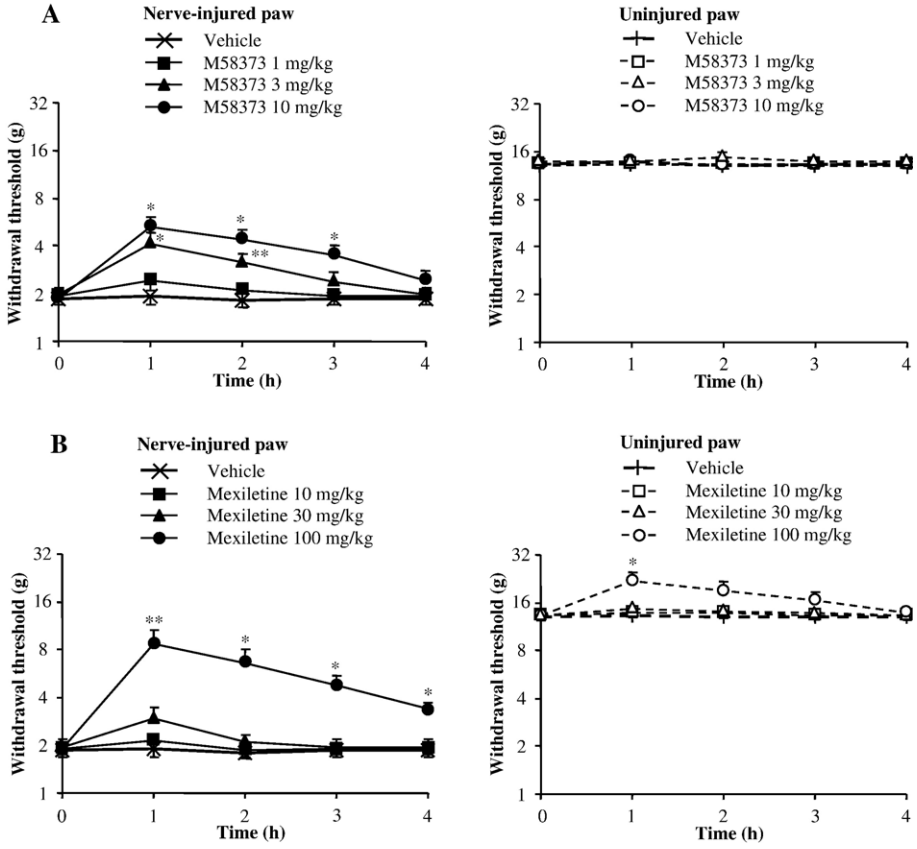

Fig. 4. Effects of M58373 and mexiletine on mechanical allodynia in the chronic constrictive injury rats. After oral treatment with a compound, the withdrawal thresholds were measured at 1, 2, 3 and 4 h. Values are expressed as means ± S.E.M. of 10 animals. *$P<0.05$, **$P<0.01$ significantly different from the pre-value (repeated measure analysis of variance on ranks (Friedman test), with Dunn's post hoc analysis).

injury rats. The withdrawal threshold of the nerve-injured paw was obviously lower than that of the uninjured paw in the vehicle-treated group, indicating mechanical allodynia. M58373 attenuated the mechanical allodynia in the nerve-injured paw in a dose-dependent manner. However, even at 10 mg/kg, M58373 did not completely reverse it. In the uninjured paw, it showed no effect (Fig. 4A). In contrast, mexiletine at 100 mg/kg almost completely reversed the mechanical allodynia, but it also increased that of the uninjured paw (Fig. 4B).

Though M58373 showed no adverse effects, mexiletine induced tonic–clonic convulsion and decreased activity level in 2 out of 10 rats at 100 mg/kg.

3.4. Effects of M58373 on heat hyperalgesia

Fig. 5 shows the effects of oral M58373 (1–10 mg/kg) and mexiletine (10–100 mg/kg) on the withdrawal latencies of the nerve-injured and uninjured paws in the chronic constrictive injury rats. The withdrawal latency of the nerve-injured paw was obviously lower than that of the uninjured paw in the vehicle-treated group, indicating heat hyperalgesia. M58373 attenuated the heat hyperalgesia in the nerve-injured paw in a dose-dependent manner. Interestingly, M58373 at 3 and 10 mg/kg completely reversed it without affecting that of the uninjured paw (Fig. 5A). In contrast, mexiletine completely reversed it without affecting that of the uninjured paw at 30 mg/kg, but it increased those of both paws over the basal value of the uninjured paw at 100 mg/kg (Fig. 5B).

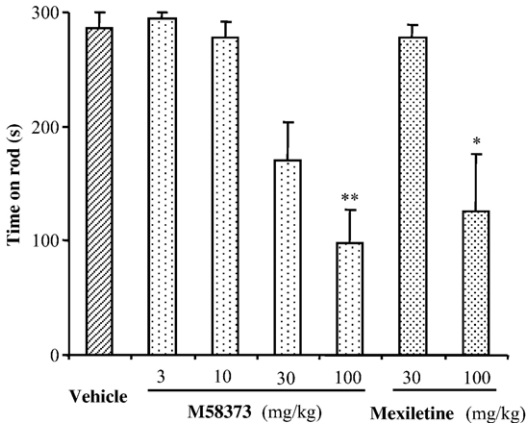

Fig. 6. Influence of M58373 and mexiletine on motor coordination in rats. Values are expressed as means±S.E.M. of 5 animals. *$P<0.05$, **$P<0.01$ significantly different from the vehicle-treated group (nonparametric Dunnett's test).

Though M58373 showed no adverse effects, mexiletine induced tonic–clonic convulsion and decreased activity level in 2 out of 10 rats at 100 mg/kg.

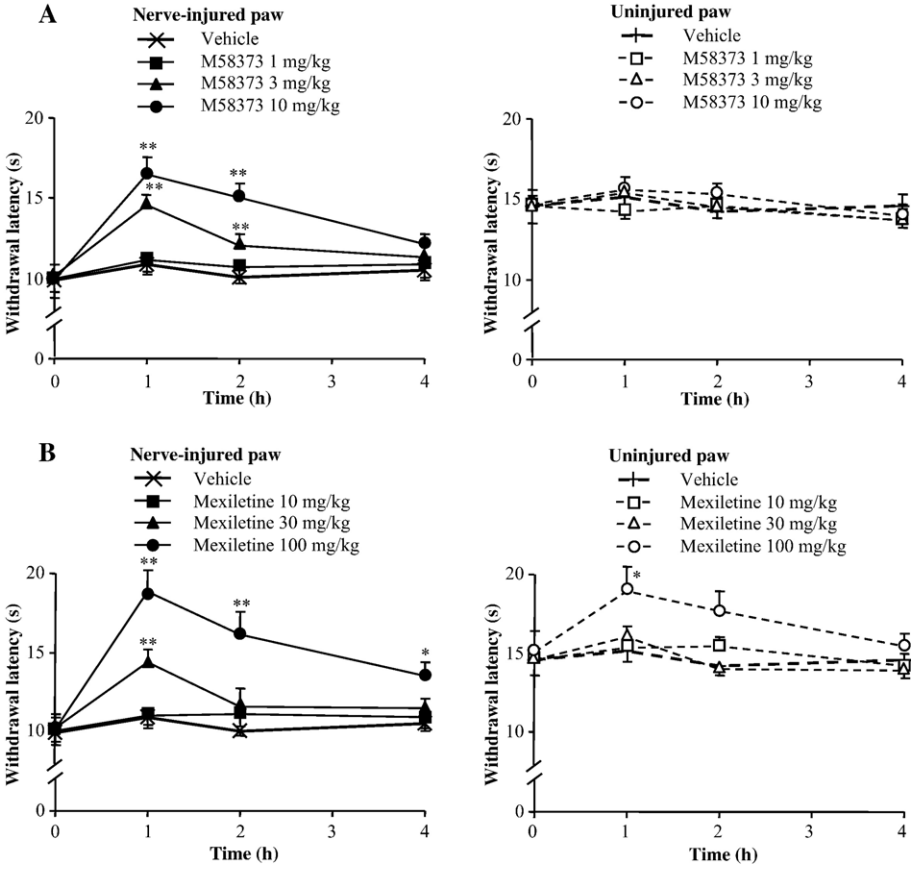

Fig. 5. Effects of M58373 and mexiletine on heat hyperalgesia in the chronic constrictive injury rats. After oral treatment with a compound, the withdrawal latencies were measured at 1, 2 and 4 h. Values are expressed as means±S.E.M. of 10 animals. *$P<0.05$, **$P<0.01$ significantly different from the pre-value (2-way repeated measure analysis of variance with Dunnett's post hoc analysis).

3.5. Influence of M58373 on motor coordination

Fig. 6 shows the influence of oral M58373 (3–100 mg/kg) and mexiletine (30 and 100 mg/kg) on motor coordination. M58373 had no influence on motor function up to 10 mg/kg, but reduced it at 30–100 mg/kg. On the other hand, mexiletine significantly reduced it at 100 mg/kg.

M58373 induced tremor and decreased activity level in 1 out of 5 rats at 30 mg/kg, and in 3 out of 5 rats at 100 mg/kg. On the other hand, mexiletine induced tonic–clonic convulsion and decreased activity level in 2 out of 5 rats at 100 mg/kg.

4. Discussion

Veratridine binds to neurotoxin receptor site 2 of voltage-gated sodium channels and enhances a persistent influx of sodium ion into the cells, resulting in the cell death in Neuro-2A cells (Hamasaki et al., 1996). Thus, we carried out veratridine-induced cytotoxicity assay as a screening test to search for a novel anti-neuropathic pain agent. Consequently, we found M58373 which potently inhibited the cell death. The affinity of compounds to site 2 of sodium channels in synaptosomes was further investigated by the binding assay using a radiolabeled neurotoxin, [^{3}H]batrachotoxinin A 20-α-benzoate, as described previously (Catterall et al., 1981). The inhibitory constant (K_i) values of M58373 (di-HCl salt) and mexiletine were 0.695 and 17.3 μM, respectively (performed by MDS Pharma Services). Previous studies have shown that mexiletine and lidocaine allosterically inhibit the neurotoxin binding to sodium channels (Postma and Catterall, 1984; Sheldon et al., 1994). However, the detailed mechanism of action of M58373 has not been fully clarified.

Veratridine also evokes the release of substance P from dorsal root ganglion cells by producing the membrane depolarization (Kessler et al., 1983). The substance P is considered to be associated with the transmission of noxious stimuli (Marx, 1979; Vachon et al., 2004). In the peripheral nervous system, substance P is localized to small C-fiber type neurons in the dorsal root ganglia which project centrally to the substantia gelatinosa (Hökfelt et al., 1977). Centrally released substance P could be associated with the facilitation of glutamate release in dorsal horn neurons (Maneuf et al., 2001). Thus, the interaction between glutamate and substance P seems to have a pivotal role in mediating the hyperexcitability of dorsal horn neurons (Urban et al., 1994). We therefore investigated the effect of M58373 on veratridine-induced release of substance P from dorsal root ganglion cells. M58373 potently inhibited the substance P release. In addition, we have confirmed that M58373 inhibits the neurotoxin binding to site 2 of sodium channels with high potency but induces no activation of the channel by itself. These data suggest that the direct and/or allosteric action of M58373 on site 2 of sodium channels contributes to its inhibitory action on the substance P release.

Formalin test is believed to be a more valid model for clinical pain than the classic tests with mechanical or heat stimuli because of the connection to tissue injury (Tjølsen et al., 1992; Abbott et al., 1995). The previous study on the sensitivity of the nociceptive test has shown that the formalin test is more sensitive than these classic tests (Le Bars et al., 2001). Therefore, we examined the effects of M58373 in the formalin test compared with those of mexiletine. Oral M58373 reduced formalin-induced nociceptive behaviors only in the late phase. In contrast, oral mexiletine reduced them in both phases as reported previously (Jett et al., 1997; Blackburn-Munro et al., 2002). It has been reported that the activity observed in the late phase is 1/2–2/3 of the magnitude achieved in the early phase (Puig and Sorkin, 1995). Since it is reasonable to hypothesize that analgesics are more effective when the neuronal activity is low, nociceptive behaviors in the late phase might be more sensitive to analgesics than those in the early phase. In this study, we have observed that oral mexiletine at 100 mg/kg induces tonic–clonic convulsion and decreases the activity level in about 20% of fasted rats. This observation is similar to a previous finding that oral mexiletine at 250 mg/kg (not 100 mg/kg) induces tonic–clonic convulsion in all non-fasted rats (Kast et al., 1982). Taken together, the central action of mexiletine at 100 mg/kg may result in potent analgesic effects on both phases in the formalin test.

The characteristics of neuropathic pain models are the increase in excitability and the generation of spontaneous action potential in primary afferent fibers at the nerve-injured site and related dorsal root ganglia. Early studies have shown that this hyperexcitability is associated with the accumulation of voltage-gated sodium channels at the nerve-injured site (England et al., 1994; Matzner and Devor, 1994). Recently, Lindia et al. (2005) have reported that multiple sodium channel subunits are likely to contribute to neuropathic pain process. The importance of sodium channels in neuropathic pain is supported by previous studies that sodium channel blockers such as tetrodotoxin and lidocaine are effective in neuropathic pain (Tanelian and Brose, 1991; Omana-Zapata et al., 1997; Kalso, 2005).

Therefore, we investigated the effects of M58373 in the neuropathic pain model compared with those of mexiletine. Oral M58373 at 3 and 10 mg/kg attenuated both mechanical allodynia and heat hyperalgesia in the nerve-injured paw without affecting the withdrawal thresholds and latencies of the uninjured paw. In contrast, oral mexiletine at 100 mg/kg increased those of both the nerve-injured and uninjured paws. These data are partly consistent with the previous reports that mexiletine attenuated allodynia and hyperalgesia in various neuropathic pain models (Jett et al., 1997; Erichsen and Blackburn-Munro, 2002; Erichsen et al., 2003; Garry et al., 2005). However, these previous reports have not shown the nonselective effect of mexiletine on the uninjured paw, presumably because its dose range is within 2-fold the effective dose (60–100 mg/kg, s.c.; 25–37.5 mg/kg, i.p.; 100 mg/kg, p.o. in non-fasted rats). In this study, we have observed that oral mexiletine at 100 mg/kg suppresses the normal responses of the uninjured paw at about 3 times the minimum effective dose which attenuates heat hyperalgesia (30–100 mg/kg, p.o. in

fasted rats). This nonselective effect may be attributed to the central action induced by oral mexiletine at 100 mg/kg in fasted rats, as mentioned above. In motor coordination test, oral M58373 reduced motor function and induced tremor at 30 mg/kg and higher, whereas oral mexiletine reduced it and induced tonic–clonic convulsion at 100 mg/kg. In addition, Igwemezie et al. (1991) have shown that the high brain distribution of mexiletine may be related to adverse effect on the central nervous system, which is associated with mexiletine therapy. Taken together, M58373 had a wider dose range showing a selective action on the nerve-injured site, compared with mexiletine. This selective action of M58373 is an interesting property, but further studies are required to elucidate its mechanisms.

In this study, both M58373 and mexiletine more preferentially attenuated heat hyperalgesia than mechanical allodynia. These findings are supported by a previous study showing that heat nociception is more sensitive to sodium channel blockers than mechanical nociception (Sakaue et al., 2004). Different processes exist in the transmission of mechanical allodynia and heat hyperalgesia. After peripheral nerve injury, A-fibers sprout from the deeper laminae into the substantia gelatinosa (lamina II) where nociceptive-specific neurons are dominant. This sprouting of A-fibers is believed to be important for the development of mechanical allodynia (Woolf and Mannion, 1999; Okamoto et al., 2001). On the other hand, noxious heat stimuli are transmitted to the spinal cord through C- and some Aδ-fibers (Yeomans et al., 1996). These neuronal fibers express transient receptor potential vanilloid 1 (TRPV1) channels which directly respond to heat stimuli. Thus, mechanical allodynia and heat hyperalgesia seem to be mainly signaled by A- and C-fibers, respectively. Both M58373 and mexiletine may preferentially attenuate C-fiber-mediated nociception because small diameter neurons are generally more susceptible to the action of sodium channel blockers (Franz and Perry, 1974).

In pharmacokinetic study, oral bioavailability of M58373 was high and its pharmacokinetic profile was linear in rats, just like mexiletine (Barrigón et al., 1983). However, this compound had an unfavorable influence on the action potential of cardiac myocytes. Consequently, we had to regard M58373 as the prototype for further research.

In summary, a putative sodium channel blocker, M58373, showed inhibitory effects on veratridine-induced release of substance P from dorsal root ganglion cells. In addition, M58373 showed an antinociceptive action in formalin test, anti-allodynic and anti-hyperalgesic effects in the neuropathic pain model. Compared with mexiletine, M58373 had a wider therapeutic dose range showing a selective action on the injured site. These results suggest that M58373 is a favorable prototype for novel anti-neuropathic pain agents.

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