Antinociceptive Activity of the N-Methyl-d-aspartate Receptor Antagonist N-(2-Indanyl)-glycinamide Hydrochloride (CHF3381) in Experimental Models of Inflammatory and Neuropathic Pain

GINO VILLETTI, MARCO BERGAMASCHI, FRANCO BASSANI, PIER TONINO BOLZONI, MARISA MAIORINO, CLAUDIO PIETRA, IVANO RONDelli, PHILIPPE CHAMIOT-CLERC, MICHELE SIMONATO, and MARIO BARBIERI

Research and Development Department, Chiesi Farmaceutici S.p.A., Parma, Italy (G.V., M.B.); Department of Neuroscience Center, University of Ferrara, Ferrara, Italy (M.S., M.B.); and Department of Clinical and Experimental Medicine, Section of Pharmacology, and Neuroscience Center, University of Ferrara, Ferrara, Italy (M.S., M.B.)

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ABSTRACT

N-(2-Indanyl)-glycinamide hydrochloride (CHF3381) is a novel low-affinity, noncompetitive N-methyl-D-aspartate receptor antagonist. The current study compared the antinociceptive effects of CHF3381 with those of gabapentin and memantine in vitro and in vivo models of pain. In isolated rat spinal cord, CHF3381 and memantine, but not gabapentin, produced similar inhibition of the wind-up phenomenon. CHF3381 suppressed the maintenance of carrageenan-induced thermal and mechanical hyperalgesia in the rat with a minimum significantly effective dose (MED) of 30 mg/kg p.o. Memantine produced a partial reversal of both thermal and mechanical hyperalgesia (MED = 10 and 15 mg/kg i.p., respectively). Gabapentin reversed mechanical hyperalgesia (MED = 10 mg/kg s.c.), but did not affect thermal hyperalgesia. In the mouse formalin test, CHF3381 and memantine preferentially inhibited the late phase (MED = 30 and 20 mg/kg i.p., respectively); gabapentin inhibited only the late phase (MED = 30 mg/kg s.c.). Unlike morphine, CHF3381 chronic administration was not accompanied by the development of tolerance in the formalin test. Furthermore, morphine tolerance did not cross-generalize to CHF3381. In rats with a sciatic nerve injury, CHF3381 relieved both cold and mechanical allodynia (MED = 100 mg/kg p.o.). In contrast, memantine was inactive. Gabapentin blocked cold allodynia (MED = 30 mg/kg s.c.), but had marginal effects on mechanical allodynia. In diabetic neuropathy, CHF3381 reversed mechanical hyperalgesia (MED = 50 mg/kg p.o.). Memantine (15 mg/kg i.p.) produced an antinociceptive effect, whereas gabapentin (100 mg/kg p.o.) had no significant effect. Thus, CHF3381 may be useful for the therapy of peripheral painful neuropathies.

Neuropathic pain results from damage to the nervous system due to many diverse processes and is an area of largely unmet therapeutic need. Tricyclic antidepressants and certain anticonvulsants are the mainstay of clinical therapy for neuropathic pain (Hempenstall and Rice, 2002). However, systematic reviews reveal that only 30 to 50% of patients suffering from neuropathic pain achieve clinically significant (>50%) pain relief with any available single therapy (Sindrup and Jensen, 1999). Furthermore, side effects of these therapies often limit their usefulness. Several neurotransmitters/neuromodulators have now been identified as being involved to varying degrees in the pain processing pathways. Among them, the amino acid glutamate, the dominant excitatory neurotransmitter of the mammalian central nervous system, seems to be a key effector in diseases reflecting long-term plastic changes in the central nervous system, such as chronic pain and pain-related neurotoxicity (Bennett, 2000). Glutamate is found in nerve terminals on spinal nociceptive neurons (Broman and Adahl, 1994) and is released in the spinal cord after stimulation of peripheral nociceptors (Ueda et al., 1994). Glutamate acts on a variety of ligand-gated ion channels or G protein-coupled metabotropic receptors. The glutamate-gated ion channels are classified in three major subclasses: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, and N-methyl-D-aspartate (NMDA) receptors. Recent preclinical studies suggest that hyperalgesia and allodynia after peripheral tissue or nerve injury may result from both an increase in transduction sensitivity of primary afferent
receptors at the site of injury and an increase in the excitability of spinal cord neurons mediated by NMDA receptors (Dickinson, 1997). In fact, NMDA antagonists have been reported to block formalin-induced pain behavior (Chaplan et al., 1997), hyperalgesia induced by chronic constriction (CCI) of the rat sciatic nerve (Quartaroli et al., 1999), and peripheral inflammation (Ren et al., 1992).

A number of human studies have evaluated the efficacy of clinically available NMDA receptor antagonists in chronic pain. This class of compounds has been shown to modulate pain and hyperalgesia, but the efficacy has been hampered by dose-limiting side effects (Fisher et al., 2000). As a consequence, the challenge has been to develop new “therapeutically safe” NMDA antagonists, specifically compounds able to prevent the NMDA receptor activation in pathological pain states without affecting the physiological activation of the receptor. Indeed, there is now considerable evidence that new moderate-affinity NMDA channel blockers, glycine\(_{\beta}\), and NR2B-selective antagonists are endowed with antinoceptive activity in animal models at doses devoid of obvious side effects (Parsons, 2001).

\(\text{N}^-(2\text{-Indanyl})\)-glycinamide hydrochloride (CHF3381) is a novel low-affinity, noncompetitive NMDA antagonist. Binding studies have shown that CHF3381 inhibits \[^{3}\text{H}]\text{-1-(2-thienyll)ciclohexyl} \text{piperidine binding to brain membranes with micromolar affinity (IC}_{50} = 8.8 \ \mu\text{M}}, in the absence of significant binding to other NMDA receptor complex subunits and/or to other glutamate receptor subtypes (Villetti et al., 2001). CHF3381 is endowed with neuroprotective activity in vitro (Gandolfi et al., 2001) and in vivo (Zucconi et al., 2002) and with anticonvulsant activity in models of generalized and partial seizures (Villetti et al., 2001). These effects were observed at doses devoid of negative side effects on motor coordination or behavior (Villetti et al., 2001). In view of the above-mentioned considerations, the capability of CHF3381 to affect the NMDA receptor complex is of interest in the context of nociception.

The present study, therefore, was designed to characterize the antihyperalgesic profile of CHF3381. The in vitro CHF3381 effect on wind-up, which is considered to be an electrophysiological phenomenon mechanistically similar to spinal central sensitization (Barbieri and Nistri, 2001), was investigated. CHF3381 activity was evaluated in in vivo models of persistent inflammatory pain and against a variety of behavioral signs in two models of neuropathic pain and compared with those of memantine and gabapentin, compounds reported to be active in pain animal models and in clinical trials (Parsons, 2001; Backonja, 2002). The possible development of tolerance to the antihyperalgesic activity of CHF3381 was also addressed.

**Materials and Methods**

**Animals**

Male Crl:CD (SD) BR rats, weighing 80 to 120 g, and male Crl:CD-1 (ICR) BR mice, weighing 14 to 16 g, were supplied from Charles River (Calco, Italy) and used in the inflammatory and chronic constriction injury models. Male Sprague-Dawley Rj:SD (IOPS Ham) rats, weighing 222 to 254 g at the beginning of the experimental phase, were also obtained from Janvier (Le Gentest, Saint-Isle, France) and used for the streptozotocin (STZ)-induced diabetic neuropathy experiment. Another group of male Sprague-Dawley Rj:SD (IOPS Han) rats, weighing in the target range of 95 to 109 g at the beginning of the experimental phase, was included as control group and did not receive STZ injection. At day 25 after STZ administration, the weight of diabetic rats was 223 \(\pm\) 24 g: the weight of the male SD rats used as control group was 299 \(\pm\) 18 g. Animals were acclimated to the laboratory environment for 5 to 7 days before entering the study. Animals were housed in standard plastic cages with wood chip bedding and were kept in temperature-controlled (22 \(\pm\) 2°C), relative humidity-controlled (55 \(\pm\) 15%), and 12-h light/dark cycle-controlled rooms with food and water available ad libitum, unless otherwise indicated. All testing procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC). In addition, we adhered to the Recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983).

**Surgery**

The CCI was carried out as described previously (Bennett and Xie, 1988). Briefly, rats (125–150 g at time of surgery) were anesthetized with sodium pentobarbital (50 mg/kg i.p., supplemented if required). The common right sciatic nerve was exposed at mid-thigh level, proximally to the sciatic trifurcation, and four ligatures (3-0 silk) with about 1-mm spacing were loosely tied around the nerve. All operations were completed by closing the muscles in layers and applying two wound clips to close the skin incision. Rats were then treated with enrofloxacin (10 mg/kg s.c.) to minimize the incidence of subsequent infection. All behavioral tests were conducted on animals at least 1 week after surgery. Neuropathic rats were able to drink and eat unaided.

**Electrophysiology**

**Wind-up.** Wind-up experiments were performed as described previously (Barbieri and Nistri, 2001). Briefly, spinal cord preparations (comprising a region from mid-thoracic level to conus medullaris) were isolated from 5- to 10-day-old Wistar rats (Harlan Italy, San Pietro al Natisone, Udine, Italy) under ether terminal anesthesia. The spinal cord was fixed to the bottom of the recording chamber and superfused (12 ml/min) with Krebs’ solution of the following composition: 113 mM NaCl, 4.5 mM KCl, 1 mM MgCl\(_2\), 7 mM K$_2$HPO\(_4\), 2 mM CaCl\(_2\), 1 mM NaH$_2$PO\(_4\), 25 mM NaHCO\(_3\), and 11 mM glucose, gassed with 95% O$_2$, 5% CO$_2$; pH 7.38 at room temperature (22°C). DC ventral root (VR) recordings (usually from L\(_2\) VRS) were obtained with glass suction microelectrodes filled with Kreb’s solution. Mono- and polysynaptic potentials were amplified and digitally stored on computer hard disk. Homolateral dorsal roots (DRs) were electrically stimulated via miniature bipolar suction electrodes. Stimulus intensity (1–20–V range; 1-ms duration) was adjusted to be at least 2 times above threshold defined as the minimum intensity to elicit a detectable polysynaptic response from the homolateral VR. The standard DR train protocol consisted of 16 train pulses (1 Hz; 5-min intertrial) that generated an incrementing depolarization (cumulative depolarization). As parameters of sensitization, we considered the amplitude of the cumulative depolarization and its rate of rise. The former was measured as difference between the baseline potential and the potential 1 s after the last stimulus; the rate of rise (expressed as percentage of control) was calculated as the increment of baseline depolarization per second, starting after the second response to a train of 16 pulses. All agents were bath-applied via the superfusing solution at the concentration mentioned in the text.

**Behavioral Tests**

**Carrageenan-Induced Thermal Hyperalgesia in the Rat.** Thermal hyperalgesia was assessed using the rat plantar test (Ugo Basile, Comerio, Italy), according to a method modified by Hargreaves et al. (1988). Briefly, rats were habituated to the apparatus that consisted of three individual Perspex boxes on a glass table. A mobile radiant heat source was located under the table and focused
onto the desired paw. Paw withdrawal latencies (PWLs) were recorded three times for both hind paws of each animal, the mean of which represented baseline for left and right hind paws. The apparatus was calibrated to give a PWL of approximately 10 s. To prevent tissue damage of the plantar zone, a 20-s cut-off was observed. After baseline testing was conducted, animals received an intraplantar injection of carrageenan (100 μl of a 20-mg/ml solution) into the right hind paw. PWLs were reassessed according to the same protocol as after 2.5 h after carrageenan, to ascertain that hyperalgesia had developed. Test compounds or the corresponding vehicle was administered 3 h after carrageenan, and PWLs were taken again at 3.5, 4, and 5 h after carrageenan.

**Carrageenan-Induced Mechanical Hyperalgesia in the Rat.** Mechanical nociceptive thresholds were measured in the rat paw pressure test (Randall and Selitto, 1957) by an analogesimter (Ugo Basile). The day before testing, rats received three training sessions. Pressure was gradually applied to the right hind paw, and paw withdrawal thresholds (PWTs) were assessed as the pressure (grams) required to elicit paw withdrawal. A cut-off point of 250 g was used to prevent any tissue damage to the paw. On the test day, two to three baseline measurements were taken before animals were administered carrageenan (100 μl of a 20-mg/ml solution) into the right hind paw. PWTs were determined again 2.5 h after carrageenan to establish that mechanical hyperalgesia had developed. Test compounds were then administered 3-h postcarrageenan, and PWTs thresholds were taken again at 3.5, 4, and 5 h postcarrageenan.

**Mouse Paw Formalin Test.** The mouse paw formalin test was a slight modification of the method originally described by Wheeler-Aceto and Cowan (1991). The day before formalin injection, mice were placed individually into clear plastic cylinders for 30 min to allow adaptation to the new environment. The day of testing, 20 μl of 1% formalin was injected into the plantar surface of the left hind paw and the animals were again placed into the cylinders on a clear plastic table for behavioral observation. A camera was placed under the table, and the behavioral experimental sessions were recorded. The amount of time in seconds that the animals spent licking and flinching the injected paw for the first 5 min (early phase), and then from 10 to 40 min (late phase) after formalin injection, was used as measurement of pain intensity. CHF3381 was administered i.p. 15 min before formalin; memantine and gabapentin were administered 1 h before formalin i.p. or s.c., respectively.

**Tolerance Studies.** The mouse paw formalin test was used to ascertain whether, after chronic treatment, tolerance develops to the antihyperalgesic activity of CHF3381 and morphine, as reported previously for the latter compound (Quarataroli et al., 1999). Furthermore, in a second study, CHF3381 was administered to animals chronically treated with morphine to establish whether morphine-induced tolerance cross-generalized with CHF3381. Evaluation of compound effects was carried out as described previously for testing after acute treatment. In the first study, mice were divided randomly into five groups (n = 12) and treated once daily for 8 days as follows: three groups with saline i.p., one group with CHF3381 60 mg/kg i.p., and one group with morphine 20 mg/kg i.p. On day 9, the groups were treated in the following manner: one saline-pretreated group was treated with saline i.p.; two saline-pretreated groups were treated either with CHF3381 30 mg/kg i.p. or with morphine 6 mg/kg i.p.; the group pretreated with CHF3381 60 mg/kg was treated with CHF3381 30 mg/kg i.p., and the group pretreated with morphine 20 mg/kg was treated with morphine 6 mg/kg i.p. CHF3381 and morphine were administered 15 and 30 min before formalin injection, respectively. In the second study, two groups of animals (n = 12) were treated once daily for 8 days with morphine 20 mg/kg i.p. Three other groups of animals (n = 12) received chronic dosing of saline i.p. On day 9, animals treated with chronic morphine received either morphine (6 mg/kg i.p., 30 min before formalin) or CHF3381 (60 mg/kg i.p., 15 min before formalin, respectively), whereas three saline-treated groups received either a similar administration of saline, morphine (6 mg/kg i.p.), or CHF3381 (60 mg/kg i.p.).

**Cold Allodynia in Neuropathic Rats.** For the measurement of cold allodynia, each rat was placed upon a metal mesh table chilled by an underlying water bath (5 ± 1°C) for a maximum of 20 s, as described previously (Bennett and Xie, 1988). Neuropathic animals responded to the contact with the cold surface by lifting the paw on the ligated side off the floor. The cold stimulus did not elicit any pain-related paw withdrawal in the sham-operated group (data not shown). For each set of experiments, animals were prescreened twice with 20-min interval between tests, to select animals displaying clear signs of allodynia, i.e., animals with a paw withdrawal latency on the ligated side of <10 s in both trials. Animals were then stratified into groups based on their mean withdrawal latency, so that the mean baseline did not differ between groups. The latency to paw withdrawal was then determined at 1, 2, and 4 h post-treatment.

**Mechanical Allodynia in Neuropathic Rats.** Rats were placed on a metal mesh table and adapted to the new environment. The mechanical stimulus was delivered to the plantar surface of both hind paws from below the floor of the test chamber by an automated testing device (dynamic plantar aesthesiometer; Ugo Basile), as described previously (Gibbs et al., 2001). A steel rod (diameter of 0.5 mm) was pushed against the hind paw with ascending force. The force went from 0 to 50 g over a 20-s period. When the animal withdrew its hind paw, the mechanical stimulus was automatically stopped, and the force at which the animal withdrew its paw was recorded to the nearest 0.1 g. Withdrawal responses were taken from four consecutive trials with at least 10 s between trials and averaged to select for animals displaying a clear reduction of the threshold (>40%) of the ligated paw in comparison with the contralateral paw. Animals were then stratified into groups based on their mean withdrawal threshold, so that the mean baseline did not differ between groups. The latency to paw withdrawal was then determined at 1, 2, and 4 h post-treatment.

**Mechanical Hyperalgesia in Rat Diabetic Neuropathy.** On day 0, diabetes was induced by the i.p. injection of STZ (75 mg/kg). Control animals received a similar administration of saline. On day 23, to confirm the presence of diabetes, glycemia was measured using blood glucose strips and the glycemia reader Reflux 5F type 970819 (Roche Diagnostics, Mannheim, Germany). On day 24, from 4:00 PM up to pain measurement, diabetic rats were submitted to a controlled diet (30% of the normal daily diet), with water available ad libitum. On day 25, distilled water, CHF3381 (25–100 mg/kg), and gabapentin (100 mg/kg) were administered by oral route and memantine (15 mg/kg) by intraperitoneal route to diabetic rats, 60 min before pain measurement. Control animals received a similar administration of distilled water. The nociceptive threshold was established in all groups using a mechanical nociceptive stimulus (paw pressure test; Randall and Selitto, 1957) by an analgesimeter (Ugo Basile). The pain threshold was measured in both hind paws.

**CHF3381 Plasma Levels in Streptozotocin-Treated Rats.** At the end of pain measurement, animals were sacrificed (under ether anesthesia) by bleeding from the abdominal aorta to determine plasma levels of CHF3381 after drug administration at different doses. Plasma was separated from blood and CHF3381 was quantitated by HPLC after liquid-liquid extraction. Briefly, 1.0 ml of plasma was added to internal standard and 0.5 ml of 0.5 M phosphate buffer, pH 11.7. Then, the samples were extracted with 6 ml of a diethyl ether/butanol mixture (80:20) containing 0.3% tetra-n-octylammonium bromide. To the organic phase, 0.2 ml of 0.1 N HCl was added and 30 μl of the acidic phase was injected into the HPLC system. CHF3381 and internal standard were separated using reverse phase chromatography, with retention time of 13.3 and 18.5 min, respectively. The mobile phase contained 15% methanol and 85% 0.5 M phosphate buffer, pH 2.7, and was pumped at a flow rate of 0.5 ml/min. The stationary phase was a C18 column (X-Terra MS, 150 × 4.6 mm, 3.5 μm; Waters, Milford, MA). Analytes were detected by using a fluorescence HPLC detector (model 474; Waters) set at
was calculated as follows: MPE

ized for each animal as maximum possible effect (MPE). This value

Only for mechanical allodynia in the CCI model data were normal-

values of the nociceptive thresholds obtained from both hind paws.

neuropathy study, nociceptive thresholds were calculated as mean

Fig. 1. Comparison of the effect on sensitization elicited by DR train

stimulation (1 Hz) by CHF3381, memantine, gabapentin, and morphine.

Effect of superfusion with CHF3381 300 μM (A), memantine 300 μM (B),
gabapentin 300 μM (C), and morphine 3 μM (D). Each panel represents
the superimposed extracellular ventral root voltage responses obtained
from the same preparation before (indicated as control) and during the
drug perfusion (indicated below the respective trace). Onset of drug
perfusion was 30 min before recording the trace. Each substance has been
tested on a different preparation. Calibration, 0.25 mV, 5 s.

Results

Wind-up. CHF3381 has been found to inhibit the wind-up

phenomenon. A representative trace of the effect observed

after a 30-min 300 μM CHF3381 perfusion is shown in Fig.

1A, in comparison with the effects of 300 μM memantine

(Fig. 1B) and of 3 μM morphine (Fig. 1D). In keeping with
data in the literature (Sivilotti et al., 1995), morphine pro-
duced a much more profound effect than CHF3381 and me-

mantine, being capable of almost completely abolishing
wind-up at the concentration tested. In contrast, gabapentin
was devoid of any effect at a concentration of 300 μM (Fig.
1C) and up to 1 mM (data not shown). The effect of CHF3381
was concentration-dependent and more potent on the cumu-

larization has been studied in detail (Fig. 2B). Nearly maxi-

mal effects were obtained within 15 to 20 min of perfusion, a
short time interval compared with memantine, as indicated
by a significantly greater effect at the 15-min time point (Fig.
2B).

Carrageenan-Induced Thermal Hyperalgesia in the
Rat. Animals showed baseline PWLs of 10 to 12 s. At 2.5 h
after carrageenan injection, the PWLs of the carrageenan-
jected paw were significantly reduced, averaging approxi-
ately 5 s in all experiments. This hyperalgesic state was
maintained in vehicle-treated animals at all time points
tested. The p.o. administration of CHF3381 (10–100 mg/kg)
3 h after carrageenan dose dependently antagonized the
maintenance of thermal hyperalgesia, with a minimum sig-
ificantly effective dose (MED) of 30 mg/kg (Fig. 3A). At the
highest dose tested, CHF3381 produced a significant and
sustained (>2 h) elevation in the PWLs of the inflamed paw.
There were no significant differences in the mean change in
thermal threshold of the noninflamed contralateral hind paw
in groups treated with any oral dose of CHF3381 (data not
shown). The s.c. administration of gabapentin (10–100 mg/
kg) was devoid of antihyperalgesic effects (Fig. 3B). After i.p.
administration, 10 mg/kg memantine partially reversed the
maintenance of thermal hyperalgesia at 4 h after carra-
geenan, whereas at the 15-mg/kg dose the effect was sig-
ificant and sustained up to 5 h after carrageenan (Fig. 3C).

Carrageenan-Induced Mechanical Hyperalgesia in
the Rat. On the test day, animals showed baseline with-
drawal thresholds of about 150 to 160 g. At 2.5 h after
carrageenan injection, the ipsilateral paw exhibited marked mechanical hyperalgesia, averaging approximately 90 g in all experiments. This hyperalgesic state was maintained in vehicle-treated animals at all of the tested time points. The p.o. administration of CHF3381 (10–200 mg/kg) at 3 h after carrageenan produced a significant and dose-dependent reversal of mechanical hyperalgesia with a MED of 30 mg/kg (Fig. 4A). The highest dose of CHF3381 produced a complete reversal of the inflammatory-induced mechanical hyperalgesia, and this effect was sustained up to 5 h after carrageenan. The s.c. administration of gabapentin (10–100 mg/kg) dose dependently antagonized the maintenance of mechanical hyperalgesia (Fig. 4B), with a MED of 10 mg/kg. The highest dose of gabapentin produced a complete reversal of the inflammatory-induced mechanical hyperalgesia. Memantine after i.p. administration partially relieved mechanical hyperalgesia at 3.5 h after carrageenan injection (MED = 15 mg/kg); however, the antihyperalgesic effect did not persist at the subsequent time points (Fig. 4C).

Mouse Paw Formalin Test. In the vehicle-treated groups, s.c. injection of 1% formalin into the left hind paw of mice induced a biphasic licking/flinching nociceptive response with an early and a late phase. CHF3381 i.p. administration resulted in a dose-dependent and significant reversal of the licking/flinching response in both phases of the formalin test (Table 1). In the late phase, all four doses of CHF3381 produced a reduction of the nociceptive response, with a MED of 30 mg/kg. The s.c. injection of gabapentin resulted in a significant and dose-dependent suppression of the late phase behavior with a MED of 30 mg/kg (Table 1) and a maximal 71% reduction observed at 300 mg/kg. Gabapentin yielded only a modest effect on the early phase behavior at the highest dose (300 mg/kg) tested ($p < 0.05$). However, at this dose some mild motor weakness was observed. The i.p. administration of memantine significantly attenuated nociceptive responding in both phases of the formalin test (Table 1), even if this compound seemed to be somewhat more potent in blocking the late phase of formalin pain. Indeed, the largest reduction in the formalin-induced pain behavior was observed at the 20-mg/kg dose (53%) in the late phase.

Tolerance Studies. Morphine (6 mg/kg i.p.) significantly attenuated basal nociceptive response in both phases of formalin test in chronic vehicle-treated animals. However, the same dose of morphine administered at day 9 in animals chronically treated with 20 mg/kg i.p. morphine failed to show such effect, indicating development of tolerance (Fig. 5A). In contrast, 30 mg/kg i.p. CHF3381 showed a comparable activity in mice given chronic treatment of either 60 mg/kg i.p. CHF3381 or vehicle, indicating lack of tolerance development (Fig. 5A). Moreover, 60 mg/kg i.p. CHF3381 still demonstrated antihyperalgesic activity in mice chronically treated with morphine, indicating that no cross-tolerance exists with morphine (Fig. 5B).

Cold Allodynia in Neuropathic Rats. At baseline, animals with CCI to the sciatic nerve displayed cold allodynia by lifting the ligated hind paw off the floor with mean baseline withdrawal latencies ranging from 3.74 ± 0.29 to 5.77 ± 0.45 s. CHF3381 (10–200 mg/kg p.o.) blocked cold allodynia with a MED of 100 mg/kg. This action was dose-dependent and maximum at 2 h after administration (5.19 ± 0.53, 7.96 ± 1.56, 9.71 ± 1.59, and 11.08 ± 1.90 s for 10, 30, 100, and 200 mg/kg, respectively, versus 4.00 ± 1.19 for the vehicle-treated group; Fig. 6A). The effect disappeared by 4 h. Gabapentin (10–100 mg/kg s.c.) blocked cold allodynia at the highest dose tested (Fig. 6B). This effect remained significant for up to 4 h (3.32 ± 0.81, 6.49 ± 1.65, and 10.69 ± 2.18 for 10, 30, and 100 mg/kg, respectively, versus 4.55 ± 1.84 for the vehicle-treated group). Memantine (5–15 mg/kg i.p.) failed to have a significant effect on the maintenance of cold allodynia (Fig. 6C). At the highest dose of memantine tested (15 mg/kg i.p.), the latency to paw withdrawal at 4 h postdose was 9.22 ± 2.33 s (versus 5.52 ± 1.38 s for the vehicle; $p > 0.05$).

Mechanical Allodynia in Neuropathic Rats. At baseline, animals with CCI to the sciatic nerve displayed allodynia in response to the mechanical stimulus with a mean withdrawal latency of the ligated paw of 22.7 ± 0.2 g; the mean withdrawal latency of the contralateral paw was 43.7 ± 0.3 g. CHF3381 (10–200 mg/kg p.o.) produced a dose-dependent antiallodynic effect, with a MED of 100 mg/kg (Fig. 7A). This effect remained significant for up to 4 h and was greatest at the 200-mg/kg dose, reaching an almost complete reversal of mechanical allodynia at 120 min after the administration (MPE = 0.76 ± 0.11). Gabapentin (10–100 mg/kg s.c.) failed to produce a significant effect on the maintenance of CCI-induced mechanical allodynia (Fig. 7B). A partial effect was observed at the 100-mg/kg dose 2 and 4 h after drug administration (MPE = 0.41 ± 0.14 and 0.41 ± 0.08, respectively). Memantine (10–15 mg/kg i.p.) was devoid of significant effects at the doses tested, maximal activity...
being observed at the 10-mg/kg dose 2 h after the administration (MPE = 0.36 ± 0.06; Fig. 7C).

Mechanical Hyperalgesia in Rat Diabetic Neuropathy. A marked and significant decrease in the nociceptive threshold was evidenced in the vehicle-treated diabetic rats (136.7 ± 18.0 g) compared with the nondiabetic group (314.2 ± 11.6 g) (Fig. 8). CHF3381 orally administered at 25 mg/kg did not significantly modify the nociceptive threshold, even though the percentage of increase reached 83% (250.0 ± 17.0 g). At 50 and 100 mg/kg, however, CHF3381 significantly increased the nociceptive threshold in diabetic rats by 134% (319.2 ± 48.5 g) and 110% (287.5 ± 20.3 g), respectively (Fig. 8). Memantine (15 mg/kg i.p.) increased in a significant manner the nociceptive threshold in diabetic rats compared...
with the vehicle-treated diabetic group (108%; 284.2 ± 22.0 g) (Fig. 8). Conversely, gabapentin did not significantly modify the nociceptive threshold, although the percentage of variation reached 68% (229.2 ± 32.3 g) (Fig. 8).

**CHF3381 Plasma Levels in Streptozotocin-Treated Rats.** CHF3381 plasma levels (expressed as means ± standard deviation) of CHF3381 measured 1 h after drug administration (at the end of pain measurement) of 25, 50, and 100 mg/kg were 469 ± 280, 1206 ± 588, and 2980 ± 1753 ng/ml, respectively. A dose-proportional concentration level was therefore found. At 50 mg/kg, levels of CHF 3381, after 1 h drug administration, were similar to those found in normal rats (Villetti et al., 2001), suggesting that the absorption and distribution kinetics were not modified by 3.5 weeks after the induction of diabetes by STZ. Because the nociceptive threshold increased from 25 to 50 mg/kg CHF3381, but not further at 100 mg/kg, it may be assumed that an intermediate value between 469 and 1206 ng/ml circulating drug level is needed to obtain a significant pharmacological effect 25 days after the induction of diabetes.

### Discussion

The present results show that CHF3381 inhibits spinal nociceptive transmission in vitro and possesses antihyperalgesic and antiallodynic actions in experimental models of inflammatory and neuropathic pain. Although the anatomical sites and the mechanisms involved in CHF3381 antinociceptive effects have not been fully investigated, it is conceivable that these effects are mediated mainly by the ability of CHF3381 to block spinal NMDA receptors. It has been recently reported that CHF3381 is a low-affinity, noncompetitive NMDA receptor antagonist (Villetti et al., 2001). This is consistent with the observation that CHF3381 suppressed spinal nociceptive transmission in vitro with a similar profile and magnitude of activity to the well established noncompetitive NMDA antagonist memantine. Furthermore, CHF3381 showed an antihyperalgesic activity in experimental pain models without affecting the physiological nociceptive threshold, in line with previous reports for NMDA receptor antagonists (Ren et al., 1992; Quartaroli et al., 1999).

The first part of this study investigated the CHF3381 effect on the wind-up phenomenon, an increased excitability of dorsal horn neurons induced by repetitive C-fiber stimulation. As observed with memantine, the most pronounced CHF3381 action was on the amplitude of cumulative depolarization. Cumulative depolarization is strongly dependent on activation of high-threshold afferents and NMDA receptors (Thompson et al., 1992). Therefore, the actions of CHF3381 and memantine on cumulative depolarization are consistent with their ability to modulate the NMDA receptor ion channel. However, high CHF3381 and memantine concentrations (300 μM) were necessary to potently inhibit cumulative depolarization. Indeed, the potency of NMDA receptor antagonists is often apparently much lower in in vitro slice preparations used for electrophysiological recordings than in isolated neurons or in membranes (Asghar et al., 2000). This likely reflects the slow penetration of lipophilic substances into the spinal cord compared with cultured neurons. It should be also pointed out that other neuromediators, besides glutamate, are critically involved in the wind-up phenomenon (Sivilotti et al., 1995). Gabapentin did not affect the wind-up response, confirming previous reports (Patel et al., 2001).

CHF3381 potently and dose dependently inhibited carrageenan-induced thermal and mechanical hyperalgesia, in agreement with studies indicating a contribution of the NMDA receptor in this model (Ren et al., 1992; Taniguchi et al., 1997). CHF3381 did not modify the physiological responses of the uninjured paw to thermal and mechanical stimuli at the doses tested. A significant antinociceptive activity was evident at doses about 10-fold below those known to disrupt rat motor performance in the rotarod test (Villetti et al., 2001). In contrast, memantine only partially reversed thermal hyperalgesia; the inhibition of mechanical hyperalgesia was transient and evident only at the highest dose tested. Gabapentin reduced mechanical hyperalgesia in a dose-dependent manner, but did not affect the maintenance of thermal hyperalgesia. Gabapentin was previously shown to be equally effective at blocking carrageenan-induced mechanical and thermal hyperalgesia (Field et al., 1997). This
study and the present one are similar with respect to doses administered, route of administration, carrageenan concentration, and subjects. Therefore, the reason for this unexpected finding is not clear. Possibilities may include distinct mechanisms underlying inflammation-induced mechanical and thermal hyperalgesia, differing painful intensities evoked by mechanical and thermal tests used as experienced by the inflamed paw, or a combination of these possibilities.

In the mouse paw formalin test, noncompetitive NMDA antagonists have been reported to preferentially alleviate pain behavior during the late phase, confirming an important role for spinal NMDA receptor in contributing to central sensitization after tissue injury (Chaplan et al., 1997). Similarly, CHF3381 abolished the late phase response (up to 99%) and, to a lesser extent, inhibited the early phase (up to 65%). A similar pattern of activity was observed for memantine, even if this compound suppressed both the early (up to 44%) and the late phase (up to 53%) of formalin-induced responses only partially. Therefore, as for the carrageenan model, these results suggest that CHF3381 inhibits spinal NMDA receptors in pain models of peripheral inflammation. However, the relative contribution of spinal NMDA receptor inhibition to CHF3381 antinociceptive effect must be further verified by comparing the present results with those for

Fig. 5. Lack of tolerance to the antihyperalgesic action of CHF3381 in the mouse paw formalin test. A, in animals chronically treated with saline or 60 mg/kg i.p. CHF3381, a challenge treatment with 30 mg/kg i.p. CHF3381 significantly attenuated basal nociceptive response in both phases. Administration of 6 mg/kg i.p. morphine at day 9, after chronic treatment with morphine (20 mg/kg i.p.), was ineffective in both phases. B, in animals chronically treated with saline or morphine 20 mg/kg i.p., a challenge treatment with 60 mg/kg i.p. CHF3381 significantly attenuated basal nociceptive response in both phases. Data are shown as the mean ± S.E.M. (n = 12). *, p < 0.05; **, p < 0.01 versus saline + saline-treated animals (one-way ANOVA followed by Dunnett’s t test).
CHF3381 administered by the intrathecal route. Gabapentin activity was in line with published data (Field et al., 1997).

The results reported here suggest that, unlike morphine, CHF3381 does not induce tolerance to its antihyperalgesic effect after chronic administration in the formalin test. Eight days of 20 mg/kg morphine administration produced significant tolerance in mice treated at day 9 with 6 mg/kg morphine. In contrast, chronic treatment with 60 mg/kg CHF3381 did not modify the day 9 antihyperalgesic activity of 30 mg/kg CHF3381. We can exclude that the lack of tolerance development after CHF3381 treatment can be attributed to the use of a low dose, because CHF3381 was chronically administered at the dose maximally active in the late phase of formalin-induced pain. The present study further demonstrates that in the doses administered, morphine tolerance dose not cross-generalize to CHF3381. Whether CHF3381 can block or delay the development of opiate toler-

Fig. 6. Effect of CHF3381 p.o. (A), gabapentin s.c. (B), and memantine i.p. (C) on cold allodynia in rats with a chronic constriction injury to the sciatic nerve. Cold allodynia was assessed by measuring the paw withdrawal latency after exposure to a cold surface kept at 5°C. Baseline measurements were taken before treatment. Animals were then treated with test compounds and withdrawal latencies were reassessed at various intervals after treatment. Data are mean ± S.E.M. (n = 10–15). *p < 0.05; **p < 0.01 versus vehicle-treated animals (one-way ANOVA followed by Dunnett’s t test).

Fig. 7. Effects of CHF3381 p.o. (A), gabapentin s.c. (B), and memantine i.p. (C) on mechanical allodynia in rats with a chronic constriction injury to the sciatic nerve. Mechanical allodynia was assessed by measuring the paw withdrawal latency after exposure to a mechanical (dynamic plantar aesthesiometer) stimulation. Baseline measurements were taken before treatment. Animals were then treated with test compounds and withdrawal latencies were reassessed at various intervals after treatment. Response obtained after treatment was normalized for each animal as MPE. Data are mean MPE ± S.E.M. (n = 10–15). *p < 0.05; **p < 0.01 versus vehicle-treated animals (one-way ANOVA followed by Dunnett’s t test).
Recent studies suggest that both hyperalgesia and allodynia in peripheral nerve-injured rats are sensitive to NMDA receptor antagonism (Chaplan et al., 1997; Quartaroli et al., 1999; Yashpal et al., 2001). In agreement with these studies, CHF3381 showed the ability to partially relieve cold and mechanical allodynia in the CCI model. These effects were dose-dependent and, for mechanical allodynia, represented a 70% recovery to physiological level of response at 100 mg/kg. No significant effect was observed on contralateral withdrawal thresholds. Interestingly, the administration of doses 3-fold higher than those endowed with antihyperalgesic activity in inflammatory pain models was necessary to relieve allodynia in peripheral nerve-injured rats. Although the reason for the different potency of CHF3381 in inflammatory and neuropathic pain models remains unclear, these results might be explained by different levels of central sensitization in these two conditions and/or a different involvement of the NMDA receptor in the maintenance of allodynia and hyperalgesia in various pain states. In our experimental conditions, we failed to demonstrate an antiallodynic efficacy of memantine. This was somewhat surprising given that this compound has been reported to attenuate mechanical allodynia in peripheral nerve-injured rats (Carlton and Hargett, 1995; Chaplan et al., 1997). As for cold allodynia, we are not aware of previous behavioral assessment of memantine effect on this parameter in the CCI model. However, memantine was ineffective in reducing cold allodynia in clinical studies (Eisenberg et al., 1998). It seems plausible that CHF3381 and memantine block the NMDA receptor via the same mechanism. It is unlikely that large differences in pharmacokinetics and tissue distribution may explain the different activity of these compounds in the CCI model and also in carrageenan-induced inflammation. It is possible that this different profile of activity arises as a result of the maximum dose of drug, which could be administered without overt side effects. Indeed, the occurrence of side effects can be expected after acute administration of memantine at doses ≥20 mg/kg i.p. (Parsons et al., 1999).

Additional activities besides the NMDA receptor antagonism may also account for the superior activity profile of CHF3381. CHF3381 behaves as a nonselective monoamine oxidase (MAO) inhibitor (IC_{50} = 7.2 μM for MAOA; IC_{50} = 60.3 μM for MAOB; Villetti et al., 2002). Such an action would activate inhibitory monoaminergic descending processes originating at supraspinal levels (Millan, 2002). Thus, this could result in an interaction with NMDA receptor antagonism for the inhibition of nociceptive transmission within the spinal cord. Indeed, MAO inhibitors have been recently reported to possess antinociceptive activity in experimental models of neuropathic pain (Apaydin et al., 2001). However, the functional significance of CHF3381 MAO inhibition in experimental pain models remains to be addressed. Contrasting results were found after gabapentin treatment. The gabapentin positive effect on cold allodynia was consistent with other reports (Hunter et al., 1997). The magnitude of this effect was comparable with that observed after CHF3381 treatment. However, we could not detect a pronounced antinociceptive effect against mechanical allodynia. To verify whether the inefficacy of gabapentin was due to inadequate dosing, we treated neuropathic rats with 300 mg/kg gabapentin and observed a complete reversal of mechanical allodynia, as reported previously (Hunter et al., 1997).

Finally, we examined the antinociceptive effect of CHF3381 in rats with STZ-induced diabetic neuropathy. We confirmed that STZ-treated rats show mechanical hyperalgesia (Courteix et al., 1993). The administration of CHF3381 and memantine restored the nociceptive threshold in diabetic rats. Our results are supported by recent data indicating that magnesium and MK-801 reverse mechanical hyperalgesia in diabetic rats (Begon et al., 2000). Together, these data suggest an important role for NMDA receptor antagonism in mediating mechanical hyperalgesia in diabetic peripheral neuropathy. A single administration of gabapentin weakly improved mechanical hyperalgesia. This observation is consistent with published data, showing that gabapentin has to be administered over an extended period to attenuate mechanical hyperalgesia in neuropathic rats (Patel et al., 2001). Interestingly, CHF3381 plasma levels achieved in diabetic rats 1 h after the administration of the 50-mg/kg p.o. dose, which yielded a maximal antinociceptive effect, were comparable with those previously observed in normal rats (Villetti et al., 2001), suggesting that the absorption and distribution kinetics of CHF3381 were not modified 3.5 weeks after the induction of diabetes with STZ.

Several clinical investigations have evaluated the activity of NMDA receptor antagonists in chronic pain patients (Fisher et al., 2000). Although results from these studies have been encouraging, unwanted side effects have hampered a wide use of this class of compounds. In this study, we provide evidence that the low-affinity, noncompetitive NMDA antagonist CHF3381 inhibits spinal nociceptive transmission in vitro and is endowed with antinociceptive activity in a number of animal models of inflammatory and neuropathic pain at doses devoid of obvious side effects (Villetti et al., 2001). In light of the current therapeutic need for neuropathic pain treatment and of the proven analgesic efficacy of NMDA antagonists in patients, it could be worth assessing the ther-
neuropathic potential of CHF3381 for the treatment of neuropathic pain in double blind placebo-controlled clinical studies.

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