EFFECTS OF A NEW POTENT ANALOG OF TOCAINIDE ON hNAV1.7 SODIUM CHANNELS AND IN VIVO NEUROPATHIC PAIN MODELS

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Abstract—The role of voltage-gated sodium channels in the transmission of neuropathic pain is well recognized. For instance, genetic evidence recently indicate that the human Nav1.7 sodium channel subtype plays a crucial role in the ability to perceive pain sensation and may represent an important target for analgesic/anti-hyperalgesic drugs. In this study a newly synthesized tocainide congener, named NeP1, was tested in vitro on recombinant hNav1.4 and hNav1.7 channels using patch-clamp technique and, in vivo, in two rat models of persistent neuropathic pain obtained either by chronic constriction injury of the sciatic nerve or by oxaliplatin treatment. NeP1 efficiently blocked hNav1.4 and hNav1.7 channels in a dose- and use-dependent manner, being by far more potent than tocainide. Importantly, the new compound displayed a remarkable use-dependent effect, which likely resulted from a very high affinity for inactivated compared to closed channels. In both models of neuropathic pain, NeP1 was greatly more potent than tocainide in reverting the reduction of pain threshold in vivo. In oxaliplatin-treated rats, NeP1 even produced greater and more durable anti-hyperalgesia than the reference drug tramadol. In addition, in vivo and in vitro studies suggest a better toxicological and pharmacokinetic profile for NeP1 compared to tocainide. Overall, these results indicate NeP1 as a new promising lead compound for further development in the treatment of chronic pain of neuropathic origin. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: analgesia, chronic pain, sodium channel blocker, use dependence, chronic constriction injury, oxaliplatin-associated pain.

Chronic neuropathic pain (NeP) is present in many neurological diseases and is frequently observed in patients receiving antitumoral chemotherapy (Kennedy, 2007). Many “classical” analgesic drugs are generally much less effective in relieving NeP, which remains an unmet medical need.

Considerable in vivo and in vitro studies indicate that voltage-gated sodium channels (VGSCs) play a key role in NeP, which begins with the aberrant firing of action potential bursts in damaged neuronal tissue (Cummins et al., 2007; Dib-Hajj et al., 2009). One of the most convincing evidence may be the total lack of pain sensitivity in humans carrying mutations in the SCN9A gene that determine a loss of function of the Nav1.7 sodium channel subtype (Cox et al., 2006). Other sodium channel subtypes are also likely important in many painful conditions, including the Nav1.3, Nav1.8, and Nav1.9 isoforms (Akopian et al., 1999; Hains et al., 2003; Priest et al., 2005; Maingret et al., 2008).

Accordingly, at least three main categories of drugs currently prescribed for treatment of NeP display significant sodium channel blockade, which are the local anesthetics, some anticonvulsants and the tricyclic antidepressants (Conte Camerino et al., 2007; Priest and Kaczkowski, 2007). Topical lidocaine is a local anesthetic approved against post-herpetic neuralgia. Intravenous lidocaine and orally-administered lidocaine congeners, mexiletine and tocainide, have proven benefits against NeP in animal models or in humans (Mao and Chen, 2000; Lindstrom and Lindblom, 1987; Challapalli et al., 2005).

The use of systemically administered sodium channel blockers is however dose-limited by a number of side effects, which can prevent patients from achieving adequate treatment (Finnerup et al., 2005). Thus mexiletine is currently proposed only as a third-line medication in NeP treatment (Dworkin et al., 2007).

At least two strategies can be employed to improve efficiency and safety of sodium channel blockers in the treatment of NeP. One may consider the development of new molecules selective for the neuronal sodium channel isoforms involved in pain transmission (Jarvis et al., 2007; Williams et al., 2007). The other may consist in the development of highly state-dependent sodium channel blockers. In over-excited neurons, the channels reside for longer durations in the inactivated state, because these tissues generate high-frequency action potential discharges. Thus, compounds that selectively block inactivated sodium channel display frequency-dependent (also called use-dependent) effect that should block signaling preferentially in damaged tissues.

In previous studies, the screening of a large number of newly-synthesized analogues of mexiletine and tocainide

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Abbreviations: CCI, chronic constriction injury; HSA, human serum albumin; IBU, ibuprofen; NeP, neuropathic pain; NeP1, 1-benzyl-N-(2,6-dimethylphenyl)-3-pyrrrolidine carboxamide; VGSC, voltage-gated sodium channel.
on voltage-gated sodium channels in skeletal muscle fibers led us to the identification of NeP1 (Fig. 1), a new tocinamide congener, with very high potency and interesting use-dependent profile (De Luca et al., 2003; Muraglia et al., 2007). Here, we examine the effects of NeP1 on human Nav1.4 and Nav1.7 sodium channels expressed in a mammalian cell line, its analgesic properties in animal models of neuropathic pain, as well as in vivo and in vitro toxicity features. Overall, the results indicate NeP1 as a good candidate for further development in neuropathic pain therapy.

EXPERIMENTAL PROCEDURES

Binding studies

The binding of NeP1 to albumin was determined by chromatography using an HPLC apparatus consisting of a Jasco PU-980 Intelligent HPLC pump (Jasco, Tokyo, JP), a Rhodyne injector system with a 20 μL loop and a Jasco MD-910 multil wavelength detector (Jasco, Tokyo). The stock solution of compound NeP1 (1 mM) was prepared in HPLC grade 1-propanol (Sigma-Aldrich, Milan, Italy) and stored at 4 °C. Work solutions were prepared daily by diluting the stock solution down to 10 μM (for determination of binding to albumin) or 20 μM (for displacement experiments) with phosphate buffer (PB) prepared adjusting at 7.4 the pH of a solution of KH2PO4 0.067 M with an equimolar solution of K2HPO4 (Carlo Erba, Milan, Italy). The mobile phases were filtered through a 0.22 μM membrane filter and degassed before their use. The drug was injected into a human serum albumin (HSA) column (50×4.6 mm2, 7 μm of size particles) (Hypersil®, ThermoQuest, Runcorn, UK). The flow rate was 1 mL/min. The chromatographic runs were monitored at 220 nm. Each measure is the average of three injections.

The drug bound fraction to albumin was determined by zonal displacement experiments. Displacement experiments were performed in order to evaluate if NeP1 binds to any of the most characterized binding sites of HSA. Chromatographic retention of the drug into the HSA column was measured in the presence of increasing concentrations of competing agents into the mobile phase. The competitors employed were sodium salicylate (final concentration of 25, 50, 100 μM), (S)-ibuprofen (final concentration of 5, 10, 20 μM) and valproate (final concentration of 1, 2, 5 mM), as marker of site I, site II and bilirubin site on the protein, respectively (Peters, 1996). The reciprocal of the k-values obtained were plotted against the concentration of displacer. The displacer affinity constant for the binding site of NeP1 was calculated by the linear relationship:

$1/k_a = V_a[I]/K_m + V_a/K_a m$

where $k_a$ is the capacity factor (as previously defined), $V_a$ is the void volume of the column, [I] is the displacer concentration in the mobile phase, $m$ is the number of moles of protein immobilized into the column, $K_a$ is the affinity constant of the displacer for the binding site of the analyte and $K_a$ is the affinity constant of the analyte for its own binding site.

Electrophysiological studies

In vitro drug testing on hNav1.4 and hNav1.7 sodium channels was performed as previously described (Desaphy et al., 2009). Briefly, HEK293 cells (a human embryonic kidney cell line) were permanently transfected with the full-length hNav1.4 cDNA sub-cloned in the pRc-CMV vector, using the calcium-phosphate precipitation method followed by clone selection with geneticin (GIBCO-Invitrogen, Italy). The full-length hNav1.7 cDNAs sub-cloned in a modified pcDNA3/pBR222 expression vector was transiently transfected in HEK293 cells together with a lower amount of cDNA encoding the plasma membrane receptor CD8. For patch-clamp recordings, 36–72 h after transfection, successfully reconnected cells were recognized using Dynal coated with anti-CD8 antibody (Dynal A.S., Oslo, Norway). Whole-cell sodium currents were recorded at room temperature (20–22 °C) using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA). Voltage-clamp protocols and data acquisition were performed with pClamp 6.0 software (Axon Instruments) through a 12-bit A-D/D-A Digitida 1200 interface (Axon Instruments). The external solution contained (mM): 150 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 5 Heps and 5 glucose; the pH was set to 7.4 with NaOH. The pipette solution contained (mM): 120 CsF, 10 CsCl, 10 NaCl, 5 EGTA, and 5 Heps; the pH was set to 7.2 with CsOH. Currents were low-pass filtered at 2 kHz (∼3 dB) by the four pole Bessel filter of the amplifier and digitized at 10–20 kHz. After rupturing the patch membrane, sodium currents were elicited by a 25 ms-long test pulse to −30 mV from a holding potential of −120 mV applied at 0.1 Hz frequency, until stabilization of current amplitude was achieved (typically 5 min). Then the drug was applied at the desired concentration around the cell through a plastic capillary, and effects of drug on sodium currents was measured first at 0.1 Hz stimulation frequency then at 10 Hz. Specific protocols were used to determine affinity constant for closed resting and inactivated channels, as described in the Results section. Little or no run-down was observed during the experiments. Analysis was performed off-line, as described in the text.

Animal care and treatment

In vivo experiments were carried out in accordance with the Animal Protection Law of the Republic of Italy. DL No. 116/1992, based on the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals involved. Male Sprague–Dawley rats of 200–220 g (Harlan, Italy) were used, except for the hole-board test which was adapted to mice (see below). Three rats were housed per cage and fed a standard laboratory diet, with tap water ad libitum for 12h12h light-dark cycles (lights on at 7:00). The cages were brought into the experimental room the day before the experiment, for acclimatization purposes. All experiments were performed between 10:00 and 15:00.

Tocainide was purchased from Tocris (Italy). Its newly synthesized congener, Nep1 (1-Benzyl-N-(2,6-dimethylphenyl)-3-pyrrolidine carboxamide), was prepared as previously described (Muraglia et al., 2007), and tramadol was purchased from Sigma-Aldrich (Milan, Italy). Other chemicals were of the highest quality.
commercially available. Drugs were dissolved in saline or carboxymethyl cellulose (CMC) solution immediately before use and injected per os by gavage. Drug concentrations were prepared so that the dose could be administered in a volume of 10 ml/kg.

Rotarod test
The apparatus consisted of a base platform and a rotating rod of 5 cm diameter with a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 50 cm in length, was divided into five equal sections by six disks, allowing the simultaneous test of up to five rats with a rod-rotating speed of 11 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. The performance time was measured before and 15, 30 and 45 min after treatment.

Hole-board test
The hole board test was performed on male Swiss albino mice (24–26 g) from Morini (Reggio Emilia, Italy). Fifteen mice were housed per cage. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner (Galeotti et al., 2008). Mice were placed on the center of the board by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous motility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

Chronic constriction injury (CCI)
A peripheral mono-neuropathy was produced in adult rats by placing loosely constrictive ligatures around the common sciatic nerve according to the method described by Bennett and Xie (1988). Rats were anaesthetized with chloral hydrate. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through sciatica’s trifurcation, about 1 cm of the nerve was freed of adhering tissue and four ligatures (3/0 silk thread) were tied loosely around the nerve, about 4 –5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted when viewed with 40× magnification. The left paw was untouched.

Oxaliplatin induced hyperalgesia
Oxaliplatin (Sequoia Research Products Ltd, Pangbourn, UK) was administrated at the dose of 2.4 mg/kg for five consecutive days every week for a total of 15 i.p. injection and a cumulative dose of 36 mg/kg, according to Cavaletti and collaborators (2001).

Paw-pressure test
The instrument exerts a force that is applied at a constant rate (32 g/s) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold is given by the force that induces the first struggling from the rat. An arbitrary cut off value of 250 g was adopted.

Cytotoxicity assay
Tocainide and NeP1 were assayed for their cytotoxic effects against HeLa (human epithelial cervical cancer cells) and MRC-5 (normal human lung fibroblasts) cell lines. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA), 50 U/mL of penicillin G and 0.05 mg/mL streptomycin (Sigma Aldrich, Milan, Italy), 2 mM glutamine (Sigma Aldrich, Milan, Italy), 0.25 μg/mL amphotericin B (Sigma Aldrich, Milan, Italy) in a humidified atmosphere and 5% CO₂. HeLa were subcultured three times a week to maintain continuous logarithmic growth. MRC-5 were grown to confluence without subculturing, while medium was renewed twice a week. Confluent cells were harvested after trypsinization using Trypsin-EDTA solution 1X (Sigma Aldrich, Milan, Italy). The MTS cytotoxicity assay (Cell Titer 96® Aq One Solution Cell Proliferation Assay, Promega) was used to screen the viability of the cells incubated with the test compounds. The assay is based on the bioreduction of MTS tetrazolium into formazan by NADH and NADPH produced by dehydrogenase enzymes only in metabolically active, viable cells (Bartrup et al., 1991). Compounds were prepared as 10 mM top stock solutions, dissolved in a mixture of DMSO and HCI (95:5), and stored at 4 °C. For each cytotoxicity assay, the cells were seeded into 96-well plates at a density of 200 cells/well for HeLa and 600 cells/well for MRC-5 and allowed 24 h to adhere before drugs were introduced at final concentration of 100 μM. The reagent (16% total well volume) was added to each well and plates were incubated at 37 °C until sufficient color development had occurred (usually 3–4 h). Purple formazan product was then measured spectrophotometrically at 490 nm. The optical density (O.D.) value of each culture is a function of the amount of formazan produced and is proportional to the number of viable cells.

Data analysis
All experimental results from in vivo tests are given as the mean±SEM. An analysis of variance ANOVA, followed by Fisher’s Protected Least Significant Difference procedure for post-hoc comparison, were used to verify significance between two means. Data were analyzed with the StatView software for the Macintosh. P-values of less than 0.05 were considered significant.

Data generated from cytotoxicity assays were analyzed using Stata for Windows version 10.0. All variables were reported as median with 25th–75th centiles. For all comparisons median test (Pearson Chi-square) was corrected for continuity.

RESULTS
Block of human voltage-gated sodium channels
NeP1 was tested in vitro on sodium currents (I_{Na}) recorded in HEK293 cells transfected with the human skeletal muscle sodium channel, hNav1.4, to allow direct comparison with other sodium channel blockers, such as mexiletine, flecainide, and orphenadrine (Desaphy et al., 2004, 2009). Sodium currents were elicited by depolarizing the cell from the holding potential (HP) of −120 to −30 mV at two stimulation frequencies, 0.1 Hz for determination of tonic block and 10 Hz for use-dependent block determination. In absence of drug, such protocols did not affect I_{Na}. Fig. 2A shows representative examples of current traces recorded before (control) and after application of tocainide. With 300 μM of tocainide, the amplitude of peak I_{Na} was reduced by 33±8% at 0.1 Hz and 66±9% at 10 Hz (n=4). A ten-fold lower concentration of NeP1 was able to produce a similar block at 0.1 Hz (35±3%, n=13) and was even more potent than tocainide at 10 Hz (88±3%, n=8) (Fig. 2B). The concentration-response curves were fitted with the first-order binding function,

\[ I_{\text{DRUG}} / I_{\text{CONTROL}} = 1 / [1 + ((\text{NeP1}) / I_{\text{C50}})^{SF}] \]
where IC_{50} (µM) is the half-maximum inhibitory concentration and SF is the slope factor (Fig. 2C). The IC_{50} values calculated at the HP of −120 mV are reported in Table 1. Compared to mexiletine, flecainide, and orphenadrine, in the same experimental conditions, tocainide was ~three to eightfold less potent at 0.1 Hz and ~5- to 14-times less potent at 10 Hz (Desaphy et al., 2001, 2004, 2009). On the other hand, NeP1 showed a similar effect to flecainide at...
from the modulated receptor hypothesis that is, attempt to estimate $K_I$ of NeP1, we used an equation derived higher than those calculated on hNav1.4 channels (Table 3A, Table 1). The huge use-dependence of NeP1 effects voltages 20 mV less negative than hNav1.4 channels (Fig. 2). Differences between the two channels may arise from secondarily affect apparent binding affinities. For in-
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The half-maximum inactivation voltage ($V_h$) were calculated accord-
ging to Fig. 3A in the same cells as those used for determination of the half-maximum inhibitory concentration ($IC_{50}$), which were calculated using equation (1) at the HP of $-120$ mV from the concentration/ response relationships shown in Fig. 2.

0.1 Hz, while it was eightfold more potent at 10 Hz, indicating a remarkable use-dependent profile. The block of sodium channels by tocainide and NeP1 was fully re-
versible (not shown).

NeP1 was tested on hNav1.7 channels, which are believed to be involved in nociception (Fig. 2C). A 30-μM concentration of NeP1 blocked hNav1.7-carried $I_{Na}$ by 14±1% at 0.1 Hz ($n=5$) and 71±4% at 10 Hz ($n=4$). The $IC_{50}$ values of NeP1 on hNav1.7 channels were slightly higher than those calculated on hNav1.4 channels (Table 1). Differences between the two channels may arise from subtle differences in the binding site or in channel gating that secondarily affect apparent binding affinities. For in-

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stance, it has been shown that hNav1.7 and hNav1.4 channels show differences in inactivation properties, which might affect drug activity (Cummins et al., 1998).

In the current study, hNav1.7 channels inactivated at voltages 20 mV less negative than hNav1.4 channels (Fig. 3A, Table 1). The huge use-dependence of NeP1 effects suggests that the drug binding may be very dependent on the channel state. According to the modulated receptor hypothesis, the more the proportion of inactivated channels at the HP, the more would be the apparent drug affinity (Desaphy et al., 2004). We calculated the affinity constants of NeP1 for closed ($K_C$) and inactivated ($K_I$) hNav1.4 and hNav1.7 channels. The $K_C$ was estimated as the $IC_{50}$ value measured by depolarizing the cells at the low 0.1 Hz frequency from an holding potential of −180 mV. At such potential, the entire population of channels are in the closed state, ready to open in response to depolarization. The calculated $K_C$ value of NeP1 was 229 μM for hNav1.4 and 246 μM for hNav1.7 channels (Fig. 3B, right column). In an attempt to estimate $K_I$ of NeP1, we used an equation derived from the modulated receptor hypothesis that is,

$$1/K_{APP} = h/K_R + (1 - h)/K_I$$

where $K_{APP}$ is the $IC_{50}$ measured at a depolarized holding potential, $h$ and $(1-h)$ are the proportion of closed and inactivated channels at this HP, respectively (Desaphy et al., 2004). The values of $K_{APP}$ were calculated at HP $= -90$ mV for hNav1.4 and HP $= -70$ mV for hNav1.7 (Fig. 3B, right column). These HP are close to resting membrane voltages in skeletal muscle and peripheral neurons, re-

spectively. Although HP was different for the two channel subtypes, effect of NeP1 was very similar with $K_{APP} = 3.8$ μM for hNav1.4 and $K_{APP} = 2.96$ μM for hNav1.7 channel. This is likely related to the different voltage-dependence of channel availability, as shown in Fig. 3A. The value for $h$ in the cells used for $K_{APP}$ determination was 0.821 for hNav1.4 at HP $= -90$ mV and 0.798 for hNav1.7 at HP $= -70$ mV. Using equation 2, the calculated $K_I$ was thus 0.69 μM for hNav1.4 and 0.60 μM for hNav1.7 channels. Thus little or no differences in $K_C$ and $K_I$ was found between channel subtypes, confirming that the channel subtype difference in $IC_{50}$ at the HP of $-120$ mV was mainly due to difference in the proportion of inactivated channels. Importantly, NeP1 displayed a remarkable high affinity for inacti-

### Table 1. Half-maximum inactivation voltage of hNav1.4 and hNav1.7 sodium channel subtypes and half-maximum inhibitory concentration of tocainide and NeP1

<table>
<thead>
<tr>
<th>Channel subtype</th>
<th>$V_h$ (mV)</th>
<th>Drug</th>
<th>$IC_{50}$ at 0.1 Hz (μM)</th>
<th>$IC_{50}$ at 10 Hz (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNav1.4</td>
<td>−78.3±0.2 (n=9)</td>
<td>Tocainide</td>
<td>699±69</td>
<td>182±24</td>
</tr>
<tr>
<td></td>
<td>−79.0±0.2 (n=15)</td>
<td>NeP1</td>
<td>67±5</td>
<td>4.9±0.3</td>
</tr>
<tr>
<td>hNav1.7</td>
<td>−73.3±0.9 (n=21)</td>
<td>NeP1</td>
<td>109±6</td>
<td>7.7±0.7</td>
</tr>
</tbody>
</table>

In vivo studies

Administration of NeP1 p.o. dose-dependently reversed the mechanical hyperalgesia in CCI rats within the range 10–60 mg/kg (Fig. 4A). NeP1 exhibited a complete anti-
hyperalgesic effect only at the dose of 60 mg/kg, 30 and 45 min after administration. Analgesic effect was ended by 60 min after drug administration. The compound did not show any analgesic efficacy in the contralateral, non-injured paw in the same range of doses (Fig. 4B). The parent drug tocainide induced an increase of pain threshold only in the injured paw at the dose of 50 mg/kg p.o. (Fig. 4A) to a level quite super-
-imposed to that obtained with 10 mg/kg of NeP1.

To verify the anti-hyperalgesic effect of NeP1 in a distinct neuropathy, the effect of the drug was examined in oxaliplatin-induced painful condition (Fig. 5). Mechanical hyperalgesia observed after chemotherapeutic treatment was reversed by NeP1 at the dose of 10 and 30 mg/kg 15, 30 and 45 min after injection. By contrast the dose of 60 mg/kg, which was fully active in CCI rats, did not show any efficacy against oxaliplatin-induced neuropathy, most probably due to compensatory mechanisms (see discus-
sion). The anti-hyperalgesic effect of NeP1 (10 and 30 mg/
kg) was greater and longer than that exerted by the reference drug tramadol (40 mg/kg). In this model, the parent drug tocainide was devoid of any activity up to the dose of 50 mg/kg. Higher doses of tocainide were not tested because the drug caused a motor coordination impairment in rotarod test starting from 70 mg/kg p.o. (Table 2), as well as a significantly reduced spontaneous motility in the hole-board test at the dose of 100 mg/kg p.o. (Table 3). In contrast, no significant impairment was observed in both tests with NeP1 up to 100 mg/kg.

**In vitro toxicological studies**

In vitro effects of NeP1 compared to tocainide on cellular viability of normal human lung fibroblasts (MRC-5) and human cervix epithelioid carcinoma cells (HeLa) was as-

essed with the MTS microassay. In three independent assays the two cell lines were incubated with the 100 μM-drugs for 3 days. As reported in Fig. 6, NeP1 did not show any appreciable cytotoxic effect, both in HeLa and
MRC-5 human cell lines, whereas tocainide significantly reduced cell viability by about 30% in both cell lines.

Drug binding to HSA

The binding of drugs to plasma proteins influences significantly the drug distribution. The characterization of drug binding to plasma and tissue proteins are then considered fundamental factors in determining the overall pharmacological activity of a drug. HSA is the most important plasma protein, which acts as a drug carrier with drug pharmacokinetic implications (Peters, 1996). As the actual volume of distribution and concentration of a drug at the receptor site are strictly related to the amount of unbound drug fraction, the study of the binding mechanism to HSA has become

![Diagram](image-url)
essential to the early characterization of the pharmacokinetic profile of new potential leads.

The bound fraction of NeP1, determined from its retention time on the HSA based column, resulted to be 97%, a value much higher to that of tocainide (54%, data not shown). Displacement chromatographic experiments were then performed using zonal elution methodology in order to examine the competition displayed between NeP1 and selected agents with a known high affinity binding site on HSA (Bertucci and Domenici, 2002). A significant competition was found between NeP1 and salicylate, a HSA site I marker (Fig. 7). The affinity constant of salicylate for HSA was $1.5 \times 10^6 \text{ M}^{-1}$ in the presence of 20 $\mu$M NeP1, which is an order of magnitude lower with respect to the reported affinity constant of salicylate (Kragh-Hansen, 1990), thereby suggesting a non-cooperative binding of NeP1 to site I on HSA. Also ibuprofen (IBU) resulted efficient in decreasing the retention time of NeP1 in a dose-dependent manner (not shown). A non-cooperative binding was suggested by the quite low value ($K_c = 6.6 \times 10^3 \text{ M}^{-1}$) of the affinity constant of IBU for HSA in presence of NeP1. A less significant interaction was observed with valproate, added to the mobile phase up to 5 mM concentration.

**DISCUSSION**

Voltage-gated sodium channels are one of the major classes of ion channels responsible for driving neuronal excitability in both the central and peripheral nervous system. As a therapeutic class, sodium channel blockers have been widely used to treat disorders where the therapeutic approach is designed to decrease cell excitability. Sodium channels expressed in primary afferent neurons have been implicated in the aberrant firing patterns that follow nerve injury.

![Fig. 4](image-url)  
**Fig. 4.** Effect of NeP1 and tocainide on peripheral monolateral neuropathy induced by chronic constriction injury of sciatic nerve in the rat. (A) Dose-dependent anti-hyperalgesic effect of NeP1 and tocainide in nerve-injured paw (dx) after p.o. administration of drugs evaluated with the mechanical paw pressure test. (B) Effect of NeP1 and tocainide in the controlateral, non-injured, paw (sx). Data are presented as paw withdrawal threshold in grams. Each data point represents the mean±SEM of five to six rats. $^* P<0.05$ versus non-operated+saline group (hyperalgesic effect), $^\wedge P<0.05$ versus operated+saline group (anti-hyperalgesic effect).

![Fig. 5](image-url)  
**Fig. 5.** Effect of NeP1 and tocainide compared to tramadol (40 mg/kg p.o.) on oxaliplatin-induced hyperalgesia in the rat paw-pressure test. Each point represents the mean±SEM of five to six rats. $^\wedge P<0.05$ versus pre-test before oxaliplatin treatment indicates hyperalgesic effect. $^* P<0.05$ versus oxaliplatin+CMC treated group shows anti-hyperalgesic effect.

**Table 2.** Effects of NeP1 compared to tocainide in the rat rotarod test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg kg$^{-1}$)</th>
<th>Before treatment</th>
<th>After treatment</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>CMC</td>
<td></td>
<td></td>
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<tr>
<td>NeP1</td>
<td>60</td>
<td>4.3±0.4</td>
<td>2.6±0.5</td>
</tr>
<tr>
<td>NeP1</td>
<td>100</td>
<td>4.0±0.4</td>
<td>2.9±0.4</td>
</tr>
<tr>
<td>Tocainide</td>
<td>50</td>
<td>4.2±0.5</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>Tocainide</td>
<td>70</td>
<td>4.3±0.4</td>
<td>3.6±0.4</td>
</tr>
</tbody>
</table>

Drugs and vehicle were administrated per os. Each value represents the mean±SEM of five to six rats. $^\wedge P<0.05$, $^* P<0.01$ compared to CMC treated rats.
injury, suggesting that blockers of these channels could prove beneficial in the treatment of neuropathic pain conditions (Cummins et al., 2007; Priest and Kaczorowski, 2007; Dib-Hajj et al., 2009). Among the sodium channel subtypes expressed in sensory neurons, human genetic evidences pointed out the hNav1.7 isoform, encoded by the SCN9A gene, as an essential contributor to pain signaling (Cox et al., 2006). Thus, Nav1.7 appears to be an outstanding "validated" pain target.

In previous studies, we explored a large number of newly-synthesized analogues of mexiletine and tocainide, with the aim at a better understanding of the drug–channel interaction and the identification of more potent and state-dependent sodium channel blockers (De Luca et al., 2000, 2003; Desaphy et al., 1999, 2001). Among these compounds, NeP1 deserved our attention because it was greatly more potent that its parent compound and other analogues, and displayed a remarkable use-dependence (De Luca et al., 2003; Muraglia et al., 2007). Tocainide belongs to the local anesthetic drugs used in the treatment of symptomatic life-threatening ventricular arrhythmias and for symptomatic treatment of skeletal muscle hyper-excitability in myotonic syndromes (Conte Camerino et al., 2007). Interestingly, analgesic effects of tocainide have been also described in humans and rats (Lindstrom and Lindblom, 1987). However, its use may be limited by severe side effects including blood dyscrasias characterized by neutropenia or aplastic anemia, suggesting a toxic effect on cell proliferation (Rawson et al., 1998). The introduction of a benzylated-β proline-like group in tocainide, as in NeP1, greatly enhanced inhibition of sodium currents recorded in native skeletal muscle fibers (De Luca et al., 2003; Muraglia et al., 2007). We now show that NeP1 is a very potent blocker of human Nav1.4 and Nav1.7 channels. Importantly, the compound was very use-dependent, being ~14 times more potent at 10 Hz compared to 0.1 Hz frequency stimulation. Compared to the well-known sodium channel blockers, mexiletine and flecainide, the IC₅₀ measured at 10 Hz was the lowest we measured so far in similar conditions (Desaphy et al., 2001, 2004). Compared to orphenadrine, an analgesic acting on various targets including hNav1.7, NeP1 was 2.5 times more potent at 10 Hz (Desaphy et al., 2009). Such a behavior may be attributable mainly to the very high affinity for inactivated sodium channels, with a Kᵢ close to 0.5 µM. It is quite probable that the benzyl group added to the amine terminal may increase interaction strength at the local anesthetic binding site, by increasing both lipofilia and hindrance at this critical pharmacophore moiety (Desaphy et al., 1999, 2001; De Luca et al., 2000). The local anesthetic binding site is well conserved among sodium channel isoforms, suggesting that NeP1 may bind significantly other channel subtypes. Thus we cannot fully exclude that analgesic action of NeP1 may derive from the block of various sodium channels involved in nociception. Recently, new compounds have been shown to display a relative selectivity toward Nav1.8 or Nav1.7 channel subtypes (Jarvis et al., 2007; Williams et al., 2007). Interestingly, all these selective compounds have shown antinociceptive activity in neuropathic models, suggesting that blocking the two channel populations may be clinically useful (Jarvis et al., 2007; London et al., 2008). Thus whether selective action on hNav1.7 or hNav1.8 may represent an actual advantage in pain therapy over unselective action on both channels (as it likely occurs with NeP1) is still unknown. In addition, it has been recently suggested that, while both V102862 and A-803467 compounds are potent inhibitors

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**Table 3.** Effects of NeP1 compared to tocainide in the mouse hole-board test

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg kg⁻¹)</th>
<th>Number of mice</th>
<th>Number of movements on plane</th>
<th>Number of head punging</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td></td>
<td>18</td>
<td>40.6±5.5</td>
<td>31.8±4.1</td>
</tr>
<tr>
<td>NeP1</td>
<td>60</td>
<td>9</td>
<td>38.6±6.3</td>
<td>29.5±3.9</td>
</tr>
<tr>
<td>NeP1</td>
<td>100</td>
<td>9</td>
<td>44.1±5.8</td>
<td>33.4±3.6</td>
</tr>
<tr>
<td>Tocainide</td>
<td>50</td>
<td>8</td>
<td>41.8±4.4</td>
<td>32.6±3.5</td>
</tr>
<tr>
<td>Tocainide</td>
<td>100</td>
<td>10</td>
<td>25.9±6.6*</td>
<td>18.4±3.4*</td>
</tr>
</tbody>
</table>

Compounds were administered per os 30 min before test. * P<0.01 compared to CMC-treated mice.

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![Graph](Image 66x124 to 286x297)

**Fig. 7.** Reciprocal plot of NeP1 displacement by salicylate [linear regression equation: y=−0.0006x+0.0394, r²=−0.8384].

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**Fig. 6.** Cytotoxicity of NeP1 and tocainide (RS-TOC) tested at the concentration of 100 µM in MRC-5 and HeLa cells. Vehicle (DMSO/HCl 0.1%) was used as control. Each experiment was performed in quadruplicate; data are expressed as median with 25th–75th centiles of three to five experiments. * P<0.001 versus respective control.
of Nav1.8, the compound V102862, rather than A-803467, may be useful as an analgesic where physiological firing frequencies are higher (Browne et al., 2009). Thus even for subtype-selective sodium channel blockers, use dependency may preserve a primary role in defining the therapeutic utility of these compounds. Altogether, these observations suggest that the strongly use dependent NeP1 may be therefore of interest in the treatment of neuropathic pain. Importantly, the huge use-dependence may also increase the safety of NeP1, allowing the compound to inhibit selectively sodium currents in cells discharging high-frequency trains of action potentials, such as nociceptive neurons during pain transmission.

In accord with the critical role of sodium channels in pain transmission, the results of the present experiments provided demonstration of an anti-hyperalgesic activity of NeP1 tested in vivo using the paw-pressure test, in two animal models of persistent peripheral neuropathic pain produced either by a chronic constriction injury to the sciatic nerve or by oxaliplatin treatment. In the former model, the nerve injury has been shown to induce dramatic changes in the expression of sodium channels, including up-regulation of the Nav1.3 channel, thereby boosting high frequency firing (Dib-Hajj et al., 1999). While the specific role of the various sodium channel subtypes in determining hyperalgesia in the CCI model remains unclear, various sodium channel blockers have been shown to reduce hyperalgesia. Accordingly, NeP1 demonstrated a pronounced anti-hyperalgesic effect. The higher dose tested (60 mg/kg) was able to produce a full anti-hyperalgesic effect, whereas the maximal tolerated dose of tocainide (50 mg/kg) displayed only partial anti-hyperalgesia. The lack of antinociceptive effect of NeP1 on the paw threshold of the controlateral uninjured paw in CCI rat is consistent with its use-dependent mechanism of action. In fact the ectopic discharge generated in the chronically damaged fibers is preferentially affected by the examined compound, with respect to the lower activity of controlateral fibers, thus discriminating between a pathological and normal impulse conduction.

Regarding oxaliplatin, peripheral neuropathy is the most severe side effect of antitumoral therapy with this drug and the most frequent dose-limiting toxicity. The ability of oxaliplatin to induce hyperalgesia cannot be explained by morphological changes of the nerve, suggesting that the drug is endowed with the property to directly interact with nerve excitability (Wilson et al., 2002). An alteration of the activation and inactivation kinetics of Na⁺ channels was indeed observed in rat sensory neurons, which probably results in sodium current enhancement (Adelsberger et al., 2000). Recently, oxaliplatin-induced hyperexcitability at motor and autonomic neuromuscular junctions was shown to occur through effects on voltage-gated sodium channels (Webster et al., 2005). In addition, the sodium channel blocker, carbamazepine, was shown to reduce the neurotoxic effects of oxaliplatin both in vitro and in vivo (Lersch et al., 2002; Webster et al., 2005). Accordingly, NeP1 showed dramatic effects against oxaliplatin-induced hyperalgesia, showing a response of similar amplitude but more durable than the reference drug tramadol used at higher concentration. In both pre-clinical and clinical studies, tramadol was shown to strongly attenuate chemotherapeutic painful condition (Xiao et al., 2008). Interestingly, although tramadol is widely considered as a weak opioid receptor agonist, it was recently shown to significantly reduce sodium currents in vitro, which may contribute to its clinical effects (Haeseler et al., 2006). It is thus predictable that the potent blocker of inactivated sodium channels, NeP1, would be useful to those patients suffering from severe and dose-limiting side effects during cytostatic therapy.

It is worth to note that the lack of effect of the higher NeP1 dose (60 mg/kg) suggests that such a dose may inactivate NeP1 itself, which may result either from metabolism induction or from compensatory mechanisms. The first mechanism is unlikely because the same dose was still effective in the CCI model. The second mechanism implies that the higher dose of NeP1 may activate cellular mechanisms (may be through modulation of other ion channels), that are able to compensate for the block of sodium channels in and only in the oxaliplatin model. Noteworthy, oxaliplatin was shown to reduce acutely potassium currents in various neuron types (Benoit et al., 2006; Wu et al., 2009). In such a case, we could speculate that an ultraer inhibition of these K⁺ currents by high doses of NeP1 would be able to increase neuron excitability in oxaliplatin-treated rats, thereby counteracting the inhibition of sodium channels.

NeP1 at anti-hyperalgesic doses produced no behavioral side effect in mice as observed in the Irwin test (data not shown). The compound did not show any impairment in the motor coordination of rats in the rota-rod test, whereas tocainide starting from the dose of 70 mg/kg caused an increase of falls. Spontaneous activity of mice evaluated in the Hole-board test was not altered by NeP1, whereas tocainide (100 mg/kg) significantly reduced spontaneous motility. Interestingly, these results suggest that NeP1 may be free of dizziness and somnolence effects, which are common side effects of analgesic sodium-channel blockers. In addition, no cytotoxic effect of NeP1 was found on the two human cell lines studied, whereas tocainide reduced cell viability in the same conditions. Such a result may suggest a lower risk of blood dyscrasias with NeP1. Other studies are being conducted to better characterize the toxicologic profile of this new compound. Finally the pharmacokinetics of the interaction between NeP1 and HSA was investigated, showing that the bound fraction of NeP1 may reach 97%, which may represent an improvement in drug pharmacokinetics with respect to tocainide.

CONCLUSION

The new compound, NeP1, is a potent blocker of inactivated hNav1.7 channels, which results in a remarkably pronounced use-dependent effect. The efficacy of NeP1 in CCI and oxaliplatin-induced hyperalgesia is in agreement with the capability exhibited by compounds with VGSC blocking property to be effective in chronic pain conditions.
of neuropathic origin. The pain relief by VGSC blockers has been linked to their ability to quench neuronal hyper-excitability in pain-sensitive sensory neurons, thereby decreasing abnormal pain sensations. Although NeP1 may not be specific for hNav1.7 VGSC subtype, the pronounced use-dependence may be significantly protective against undesired effects on healthy excitable tissues. First results suggest good toxicological profile and pharmacokinetics, indicating that NeP1 constitute a good candidate for further optimization toward clinical trials.

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