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A nitric oxide (NO)-releasing derivative of gabapentin, NCX 8001, alleviates neuropathic pain-like behavior after spinal cord and peripheral nerve injury

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1 Nitric oxide (NO) participates, at least in part, to the establishment and maintenance of pain after nerve injury. Therefore, drugs that target the NO/cGMP signaling pathway are of interest for the treatment of human neuropathic pain. Various compounds endowed with NO-releasing properties modulate the expression and function of inducible nitric oxide synthase (iNOS), the key enzyme responsible for sustained NO production under pathological conditions including neuropathic pain.

2 With this background, we synthesized a new chemical entity, [1-(aminomethyl)cyclohexane acetic acid 3-(nitroxymethyl)phenyl ester] NCX8001, which has a NO-releasing moiety bound to gabapentin, a drug currently used for the clinical management of neuropathic pain. We examined the pharmacological profile of this drug with respect to its NO-releasing properties *in vitro* as well as to its efficacy in treating neuropathic pain conditions (allodynia) consequent to experimental sciatic nerve or spinal cord injuries.

3 NCX8001 (1–30 μM) released physiologically relevant concentrations of NO as it induced a concentration-dependent activation of soluble guanylyl cyclase ($EC_{50} = 5.6\,\mu$M) and produced consistent vasorelaxant effects in noradrenaline-precontracted rabbit aortic rings ($IC_{50} = 1.4\,\mu$M).

4 NCX8001, but not gabapentin, counteracted in a concentration-dependent fashion lipopolysaccharide-induced overexpression and function of iNOS in RAW264.7 macrophages cell line. Furthermore, NCX8001 also inhibited the release of tumor necrosis factor alpha (TNFα) from stimulated RAW264.7 cells.

5 NCX8001 (28–280 μmol kg^{-1}, i.p.) reduced the allodynic responses of spinal cord injured rats in a dose-dependent fashion while lacking sedative or motor effects. In contrast, gabapentin (170–580 μmol kg^{-1}, i.p.) resulted less effective and elicited marked side effects.

6 NCX8001 alleviated the allodynia-like responses of rats to innocuous mechanical or cold stimulation following lesion of the sciatic nerve. This effect was not shared by equimolar doses of gabapentin.

7 Potentially due to the slow releasing kinetics of NO, NCX8001 alleviated pain-like behaviors in two rat models of neuropathic pain in a fashion that is superior to its parent counterpart gabapentin. This new gabapentin derivative, whose mechanism deserves to be explored further, offers new hopes to the treatment of human neuropathic pain.

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Keywords: Analgesia; neuropathic pain; gabapentin; nitric oxide; NCX8001

Abbreviations: IBMX, isomethyl-butyl-xanthine; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NA, noradrenaline; NCX8001, [1-(aminomethyl)cyclohexane acetic acid 3-(nitroxymethyl)phenyl ester; NO, nitric oxide; ODQ, [1H-[1,2,4]oxadiazolo[4,3-α]quinoxaline-1-one]; PC12, pheochromocytoma cell line; sGC, soluble guanylyl cyclase; TNFα, tumor necrosis factor alpha; YC-1, [3-(5′-hydroxymethyl-2′furyl)-1-benzylindazole]

Introduction

Neuropathic pain after injury or dysfunction of the peripheral and central nervous system remains a difficult clinical problem for which effective treatments are lacking (Bennett, 1994). Anticonvulsants, such as carbamazepine or phenytoin, have been traditionally used for the management of neuropathic pain. However, the efficacy of this class of drugs has not been unequivocally established and their use has often been associated with numerous side effects (Sindrup & Jensen, 1999; Jensen, 2002). More recently, some of the newer anticonvulsants, in particular gabapentin and to a lesser extent topiramate and lamotrigine, received increased attention as analgesics for treating neuropathic pain (Jensen, 2002). Gabapentin, a structural analogue of γ-aminobutyric acid (GABA), has been found to exert significant analgesic effects in several randomized, placebo-controlled, double-blind clinical trials in postherpetic neuralgia and painful diabetic neuropathy (Backonja *et al*., 1998; Rowbotham *et al*., 1998;

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Rice *et al*., 2001; Serpell *et al*., 2002; Backonja & Glanzman, 2003). Additional open labeled trials or case reports suggested that gabapentin may also be useful in other types of neuropathic pain, including that occurring after injury of the central nervous system which is known to be particularly resistant to conventional pharmacological treatments (see Nicholson, 2000; Rose & Kam, 2002 for review). It is worth noting, however, that despite the progress made with these compounds, neuropathic pain remains undertreated and in many patients gabapentin does not provide adequate pain relief. For example, in a recently published multicenter study, gabapentin produced more than 50% pain relief only in 34% of patients with postherpetic neuralgia (Rice *et al*., 2001). Similarly, another recently published trial examining the effect of gabapentin on neuropathic pain syndromes showed that gabapentin produced more than 50% reduction of allodynia in only 23% of patients (vs 15% by placebo) (Serpell *et al*., 2002). Thus, ways to improve the efficacy and potency of gabapentin are desirable.

As in many other body functions, nitric oxide (NO) plays important and complex roles in nociceptive transmission and modulation. While it has been shown that NO is involved in generating spinal cord hyperexcitability and hyperalgesia (Meller & Gebhart, 1993), activation of the NO/cGMP signaling pathway has been shown to account, at least in part, for the analgesia produced by various drugs, particularly that of those acting at peripheral sites (Duarte & Ferreira, 1992; Duarte *et al*., 1992; Chiari *et al*., 2000; Jain *et al*., 2001; Lazaro-Ibanez *et al*., 2001; Herrero *et al*., 2003). Furthermore, local application of NO-releasing drugs exerted antiallodynic-like responses in human diabetic neuropathies (Yuen *et al*., 2002).

Peripheral nerve injury induces an up-regulation of neuronal nitric oxide synthase (nNOS) in spinal cord sensory neurons, suggesting a possible involvement of NO in the neuroplastic changes consequent to the establishment and maintenance of neuropathic pain states (Zhang *et al*., 1993; Verge *et al*., 1994; Luo *et al*., 1999; Yonehara *et al*., 2003). Furthermore, neuropathic pain consequent to peripheral nerve injury has often been associated with local inflammation and over-expression of inducible nitric oxide synthase (iNOS) as well as of various inflammatory cytokines in locally recruited macrophages and Schwann cells (Levy *et al*., 1999; Wagner & Myers, 1996) that further suggest a pivotal role of NO in hyperexcitability and pain perception.

Results from functional studies are, however, controversial in that both NOS inhibitors and NO donors appear to reduce neuropathic pain-like behaviors after nerve injury (Meller *et al*., 1992; Yamamoto & Shimoyama, 1995; Hao & Xu, 1996a; Yoon *et al*., 1998; Luo *et al*., 1999; Li *et al*., 2000).

Over the last few years, a variety of well-established drugs have been improved for safety and activity thanks to the incorporation of a NO-releasing moiety in their chemical structure (Paul-Clark *et al*., 2000; Keeble & Moore, 2002; Romero-Sandoval *et al*., 2002; Chiroli *et al*., 2003). Thus, we hypothesized that the introduction of the NO-releasing moiety to the backbone of gabapentin might ultimately result in an improved therapeutic profile of the parent drug.

Here, we report the *in vitro* pharmacological characterization of the prototype new drug, NCX8001, 1-(aminomethyl)-cyclohexane acetic acid 3-(nitroxymethyl)phenyl ester, with respect to its NO-releasing properties as measured by cGMP accumulation in adrenal medullar pheochromocytoma cell line (PC12 cells) and vasorelaxant effects in noradrenaline pre-contracted aortic rings. Further, we compared the antiallodynic efficacy of this drug *in vivo* in two well-established rat models of neuropathic pain, namely, ischemic spinal cord injury (Xu *et al*., 1992) and partial ischemic sciatic nerve injury (Kupers *et al*., 1998) with that of the parent drug, gabapentin.

Finally, we investigated whether NCX8001 modulates the inflammatory pathway by evaluating the effects of this drug on lipopolysaccharide (LPS)-induced overexpression and function of iNOS as well as of the proinflammatory mediator, TNFα, in RAW 264.7 murine macrophages cell line.

Methods

Animals and drugs

Vasorelaxant properties of gabapentin, NCX8001 or vehicle were determined on isolated aortic rings from male New Zealand rabbits weighing 1.8–2.0 kg. Antiallodynic effects of these drugs were evaluated on male and female Sprague–Dawley rats (Möllegård, Denmark) weighing 200–250 g at the start of the experiments. All experimental procedures were approved by the local research ethics committee. Ischemic injury to the sciatic nerve and spinal cord was induced by a photochemical technique.

The drug NCX8001 [1-(aminomethyl)cyclohexane acetic acid 3-(nitroxymethyl)phenyl ester; MW 358.83], was synthesized by grafting the organic nitrate moiety onto the carboxylic group of gabapentin. Gabapentin [1-(aminomethyl)cyclopentane acetic acid; MW 171.24], was from commercial sources (Medichem, Girona, Spain). The guanylyl cyclase (sGS) inhibitor, ODQ [1H-[1,2,4]oxadiazolo[4,3-α]quinoxaline-1-one], the sGC NO-independent stimulator YC-1 [3-(5′-hydroxymethyl-2′furyl)-1-benzylindazole] as well as noradrenaline (NA) were obtained from Sigma BRL (St Louis, MO, U.S.A.).

NCX8001 was dissolved in a vehicle containing 40% DMSO and 60% Cremophore El and administered to the animals at 1 ml kg^{-1} of body weight. Gabapentin was dissolved in physiological saline and administered at similar volume as NCX8001.

Evaluation of NO-mediated activity

Stimulation of cGMP in cell cultures Undifferentiated pheochromocytoma (PC12) cell line was maintained in culture according to published protocols. Briefly, the cells were grown in DMEM supplemented with 5% fetal bovine serum, 10% horse serum and 50 μg ml^{-1} of penicillin/streptomycin solution at 37°C onto poly-L-lysine-coated 96-multiwell plastic culture dishes at the initial density of about 40,000 cells well^{-1}. The cells were allowed to grow for 3 days following plating. Then, the culture medium was replaced with a fresh one and the cells kept under the same conditions for additional 24 h prior to experiments.

The day of the experiment, the monolayer cells were washed twice with Hank's balanced salt solution (Invitrogen-Life Sciences) enriched with 10 mM HEPES, 5 mM $MgCl_2$ and 0.05% ascorbic acid at the final pH of 7.4 and preincubated for 10 min at 37°C onto a floating water bath. At the end of the preincubation period, the buffer was quickly removed and

fresh buffer previously equilibrated at 37°C containing 100 μM of the phosphodiesterase inhibitor, isomethyl-butyl-xanthine (IBMX) and the test drugs were added at the appropriate concentration to the cells for additional 45 min. The reaction was finally ended by the removal of the incubating buffer followed by the addition of 50 μl well^{-1} of 100% ice-cold ethanol. The plate was then dried under hot air steam and the cell residues dissolved, extracted and analyzed using commercially available cyclic GMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.).

Vasorelaxant effects in rabbit aorta The rabbits were anesthetized with thiopental-sodium (50 mg kg^{-1}, i.v.) and killed by exsanguinations. Then, the thorax was opened and the aorta dissected. Single-ring preparations (4 mm in length) of thoracic aorta were set up in physiological salt solution (PSS) containing NaCl 130 mM, $NaHCO_3$ 14.9 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, HEPES 10 mM, $CaCl_2$ 2.5 mM, ascorbic acid 170 mM and glucose 11.1 mM at the final pH of 7.4. The solution was constantly oxygenated with a mixture of O_2 : CO_2 (95 : 5%) and kept at 37°C throughout the experimental period. Isometric tension was recorded with a Grass transducer (Grass FT03) attached to a BIOPAC MP150 System (Wanstall *et al*., 2001).

The ring preparations were allowed to equilibrate for 1 h and then contracted submaximally with noradrenaline (NA; 1 μM) for at least two consecutive min so that a stable contraction was reached. Thereafter, precontracted rings were exposed to increasing concentrations of either gabapentin, NCX8001 or vehicle in the presence or absence of the sGS inhibitor, [1H-[1,2,4]oxadiazolo[4,3-α]quinoxaline-1-one] (ODQ; 10 μM) to obtain the respective cumulative concentration–response curves. The time intervals between doses were based on the time needed to reach a full response. Each arterial ring was exposed to only one combination of inhibitor and vasorelaxant agent.

Responses to relaxing agents were expressed as a percentage of residual contraction and plotted against concentration of test compounds. The IC_{50} values (where IC_{50} is the concentration producing 50% of the maximum relaxation to the test compound) were interpolated from these plots.

Effects on LPS-elicited activation of proinflammatory mediators in murine macrophage RAW264.7 cell line The murine macrophage cell line, RAW264.7 was used in this set of experiments. The cells were maintained in culture in DMEM supplemented with 10% fetal bovine serum (FBS) in the presence of 50 μg ml^{-1} of gentamycin. The day before the experiments, the cells were subcultured in either six-multiwell plates (iNOS expression experiments) or 96-multiwell plates (nitrate and tumor necrosis factor alpha (TNFα) accumulation experiments) at the initial density of 30,000 cells per cm^2 and exposed for 24 h to culturing medium enriched with only 0.4% FBS. On the day of the experiments, the cells were exposed to increasing concentrations (1–1000 ng) of LPS for 4, 8 and 16 h in presence or absence of either vehicle, NCX8001 or gabapentin. At the end of the incubation period the supernatant was collected and frozen for nitrate and TNFα measurements. The monolayer cells were either used to assess cell viability (MTT assay) or harvested for determination of iNOS protein expression by means of Western blot analysis. Nitrate accumulation in the culturing media was determined according to published protocols. Briefly, 100 μl of the supernatants were first reacted with 50 μl of 1% sulfanilamide dissolved in 5% H_3PO_3 followed by the addition of 100 μl of *N*-(1-naphtyl)ethylenediamine 0.15% solution. The quantitative assessment of nitrate accumulation was estimated by spectroscopic determination of the absorption of each individual sample at 540 nm wavelength. Similarly, the quantitative assessment of TNFα in the culture medium was performed using the ELISA method (R&D System, Minneapolis, MN, U.S.A.).

In selected experiments, the RAW264.7 monolayer cells were harvested in lyses buffer (Tris/HCl 20 mM, CHAPS 1%, EDTA 1 mM, DTT 1 mM, leupeptin 1 μg ml^{-1}, PMSF 1 mM) and processed for Western blot analysis. Briefly, the protein lysate (about 30 μg) was diluted 1 : 1 with the Lemmli reagent (final concentration: sodium dodecyl sulfate, 1%; glycerol, 10% v v^{-1}; bromophenol blue, 0.5%) in presence of 2% v v^{-1} β-mercaptoethanol, heated at 85 °C for 5 min and loaded onto 12% SDS acrylamide : bis-acrylamide gel. Protein transfer on polyvinylidene difluoride membranes was performed at 200 mA for 2 h. The membranes were then saturated with 5% nonfat dry milk and exposed to specific polyclonal antibodies (iNOS, 1 : 2000; β-actin 1 : 5000) for 2 h at room temperature. After thorough washing (Tris/HCl 0.42% pH 7.4 containing 0.1% Tween 20) the membranes were exposed to an anti-rabbit IgG antibody conjugated with horse radish peroxidase (1 : 10000) for 45 min at room temperature. Immunoreactive bands were detected using the ECL detection kit (Amersham) and analyzed using a computer-based densitometry NIH image program.

Photochemically induced ischemic spinal cord injury

Ischemic spinal cord injury was produced in female SD rat weighing 200 g according to methods described elsewhere (Xu *et al*., 1992). Specifically, the exposed spinal cord was irradiated for a total of 10 min with a laser beam at 514 nM wavelength and an output intensity of 0.16 W. We have previously reported that a subset of spinally injured rats developed a chronic pain syndrome, including marked mechanical and cold allodynia. Rats injured some months previously (5–6 months) were used in the study. Vocalization threshold to graded mechanical touch/pressure was tested with von Frey hairs. During testing the rats were gently restrained in a standing position and the von Frey hair was pushed onto the skin until the filament became bent. The frequency of stimulation was about 1 s^{-1} and at each intensity the stimuli were applied 5–10 times. The intensity of stimulation that induced consistent vocalization (>75% response rate) was considered as pain threshold.

Responses to cold was tested with ethyl chloride spray (about 50 μl) applied to the shaved allodynic skin area. The response was graded with a score of 0 = no observable response; 1 = localized response (skin twitch and contraction), no vocalization; 2 = transient vocalization, moderate struggle and 3 = sustained vocalization and aggression.

The motor performance was evaluated using a combined behavioral score (CBS) (Hao & Xu, 1996b). Specifically, the CBS assigns a weight to each test and combines them into one total score that represents the degree of motor impairments. The animals were tested at constant time intervals (every 60 min) starting immediately before the administration of the

drugs (time 0) that was taken as baseline motor performance and for 4 hours thereafter. Data are presented as percentage changes from the baseline value. In addition to the effect on motor performance, the animals were also inspected for potential sedative effects of drugs by observing the vigilance of the rats as well as their responses to auditory stimuli.

Photochemically induced ischemic peripheral nerve injury

The left sciatic nerve of male SD rats was exposed under chloral hydrate (300 mg kg^{-1}, i.p.) anesthesia and then photochemically injured by irradiating the nerve for 2 min with a laser beam at 514 nM and an output intensity of 0.16 W (Kupers *et al*., 1998).

Behavioral tests were conducted first daily and then weekly after irradiation. The rats were put in chambers with metal mesh floors. Von Frey hairs were used to assess mechanical allodynia. They were applied in ascending order on the plantar surface of the hind paw at a frequency of 1 s^{-1}. The lowest force at which the animal withdrew the paw in at least two of three trials was taken as mechanical threshold. The highest intensity of mechanical stimulation was 73 g as stronger stimuli lifted the paw.

The response to cold was tested with ethyl chloride, which was briefly (< 1 s) sprayed on the plantar surface of the hind paw. The responses were scored as the following: 0 = no response, 1 = startle-like response, no hind paw withdrawal, 2 = brief withdrawal of the stimulated hind paw, 3 = sustained or repeated withdrawal of the stimulated hind paw, brief licking or shaking and 4 = prolonged withdrawal, shaking and licking of the hind paws, vocalization and generalized aversive reactions.

Statistics

The data from cGMP, vasorelaxation, nitrate and TNFα accumulation experiments are presented as mean ± s.e.m. and analyzed with Dunnett's test. The data referring to the effects of acute and chronic drug treatment with the von Frey hair test and cold test are expressed as median ± median absolute deviation (M.A.D.) and analyzed with Wilcoxon signed-ranks test. The other data are presented as mean ± s.e.m. and analyzed with ANOVA followed by paired *t*-test.

Results

Measurement of NO-mediated activity

cGMP levels in PC 12 cells We determined the extent of cGMP accumulation elicited by the exposure of undifferentiated PC12 cells to increasing concentrations of either gabapentin or NCX8001. As shown in Figure 1a, NCX8001 elicited, albeit modest, significant increase in cGMP levels thus suggesting that it might release NO with rather slow kinetics. Similar effects were also observed when we used human platelets as cell system (data not shown). It has recently been reported that the NO-independent stimulator of soluble guanylyl cyclase, YC-1, stabilizes the binding of NO to the prosthetic group of the enzyme and, consequently, potentiates its cGMP synthesizing properties (Friebe *et al*., 1998; Galle *et al*., 1999). Thus, we used YC-1 as a pharmacological tool to study the effects elicited by the relatively low amount of NO released over time by NCX8001. The exposure of PC12 cells to different concentrations of YC-1 resulted in an increase of cGMP with an estimated EC_{50} of 1.9 ± 0.2 μM and a maximal response that was evident at 10 μM (Figure 1b). The concomitant application of different concentrations of NCX8001 (1, 3, 10 and 30 μM) to YC-1 (30 μM) elicited cGMP accumulation in a concentration-dependent fashion that was much greater than either drug alone (Figure 1b and c). Interestingly, the effective concentration of NCX8001 did not change significantly regardless of the presence of YC-1 during the experiments. As expected, these effects were not shared by the parent drug gabapentin suggesting that they were dependent on the release of exogenous NO.

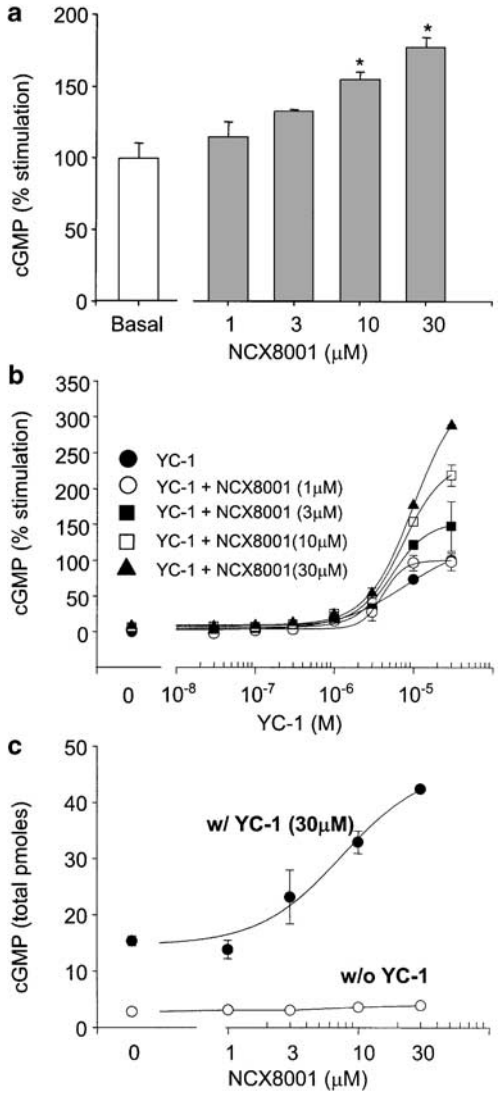

Figure 1 cGMP accumulation in rat undifferentiated pheochromocytoma cell line (PC12) following treatment with NCX8001. Panel (a) depicts the effects of either vehicle (basal) or increasing concentrations of NCX8001 applied directly to the cells. Panel (b) shows the concentration–response profile of NCX8001 obtained in presence or absence of increasing concentrations of the NO-independent activator of the soluble guanylyl cyclase YC-1. Panel (c) depicts the regression analysis of the data obtained with increasing concentrations of NCX8001 in presence of maximal effective concentration of YC-1. The latter was used to determine the EC_{50} values.

Vasorelaxant activity in rabbit aorta Physiologic concentrations of NO released from slow NO-releasing compounds such as NCX8001 can be detected using noradrenaline (NA)-precontracted rabbit aortic rings that are known to respond with relaxation to activation of the NO/cGMP signaling pathway.

Application of a submaximally effective concentration of NA (1 μM) to aortic rings elicited a measurable contraction, which reached a stable plateau thereafter. Under these experimental conditions, the exposure to vehicle or gabapentin at concentrations up to 100 μM did not induce any appreciable relaxation of NA-precontracted aortic rings (Figure 2a). Conversely, NCX8001 elicited concentration-dependent effects from 10 nM to 10 μM with an estimated IC_{50} of 1.4 ± 0.05 μM (Figure 2a). These effects were retained in the absence of functional endothelium (data not shown) thus suggesting that they do not depend on endogenous release of NO. Additional experiments were performed in the presence of the guanylyl cyclase inhibitor, ODQ, which was applied at 10 μM to precontracted aortic rings for 20 min prior to their exposure to either vehicle, gabapentin or NCX8001. As shown in Figure 2b, ODQ did not significantly alter either the basal responses or those recorded following the application of gabapentin but it virtually abolished the vasorelaxant properties of a fully effective concentration (10 μM) of NCX8001 (Figure 2b). Overall, these experiments indicate that NCX8001 is capable of releasing physiologically relevant amount of NO that could, in turn, activate the cGMP signaling pathway.

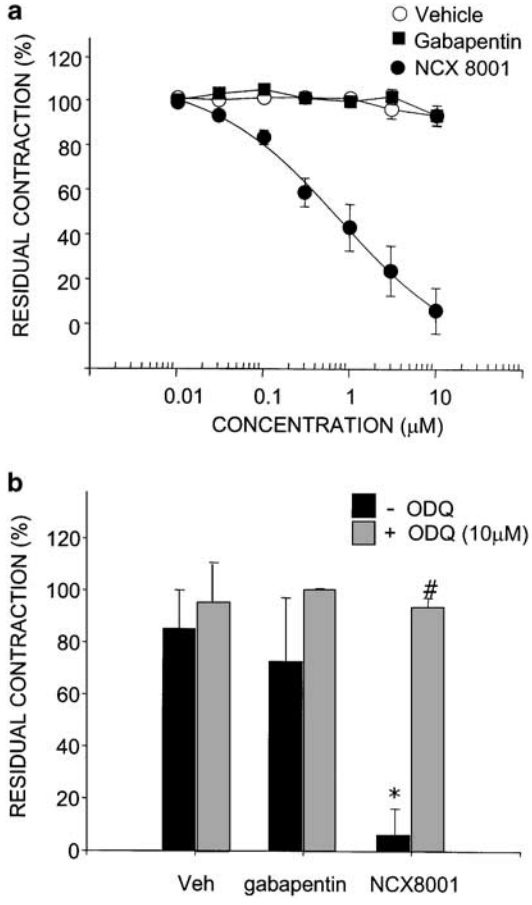

Figure 2 Vasorelaxant effects of vehicle ($n=3$), gabapentin ($n=3$) or NCX8001 ($n=5$) in NA-precontracted aortic rings (a). Panel (b) shows the residual contraction recorded following the application of either vehicle, gabapentin (10 μM) or NCX8001 (10 μM) in presence or absence of the guanylyl cyclase inhibitor, ODQ. $^{*}P<0.05$ compared to respective control group and $^{\#}P<0.05$ compared to NCX8001 in absence of ODQ, Dunnett's test.

Modulation of LPS-elicited activation of proinflammatory mediators in murine macrophage RAW 264.7 cell line Having established the NO-releasing properties of NCX8001, we next sought to determine whether this compound, like other slow NO-releasing drugs (Fiorucci *et al*., 2002), modulates the expression and function of iNOS as well as of TNFα production following the exposure of RAW 264.7 cells to the proinflammatory bacterial toxin, LPS. The exposure of cells to LPS (1000 ng ml^{-1}) elicited a time-dependent increase of TNFα in the culture media that reached a steady state within 6–8 h and remained stable until after 16 h. NCX8001 but not gabapentin inhibited in a concentration-dependent fashion the effects of LPS (Figure 3). Similarly, prolonged exposure (16 h) to increasing concentrations (1–1000 ng ml^{-1}) of LPS upregulated the expression of iNOS and, consequently, increased the content of nitrate (one of the inactive metabolites of NO) in the culturing media in a concentration-dependent fashion (data not shown). The application of NCX8001 (1–100 μM) resulted in a concentration-dependent inhibition of nitrate accumulation (Figure 4a) as well as of iNOS expression (Figure 4b and c) elicited by the maximal effective concentration (1000 ng ml^{-1}) of LPS. Conversely, gabapentin did not induce any appreciable effects on either parameters analyzed in these studies (Figure 4a–c). None of the treatments affected cell viability.

Spinal cord injury

The irradiation of the lumbar segment of spinal cord produced reproducible allodynic/hyperalgesic responses that were fully evident after several weeks postlesion and remained stable thereafter.

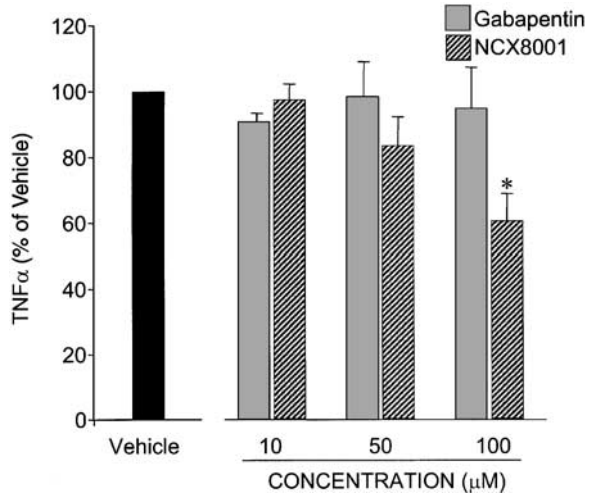

Figure 3 Effects of vehicle, gabapentin or NCX8001 on LPS-induced TNFα accumulation in RAW264.7 cells. $^{*}P<0.05$ compared to respective vehicle or gabapentin.

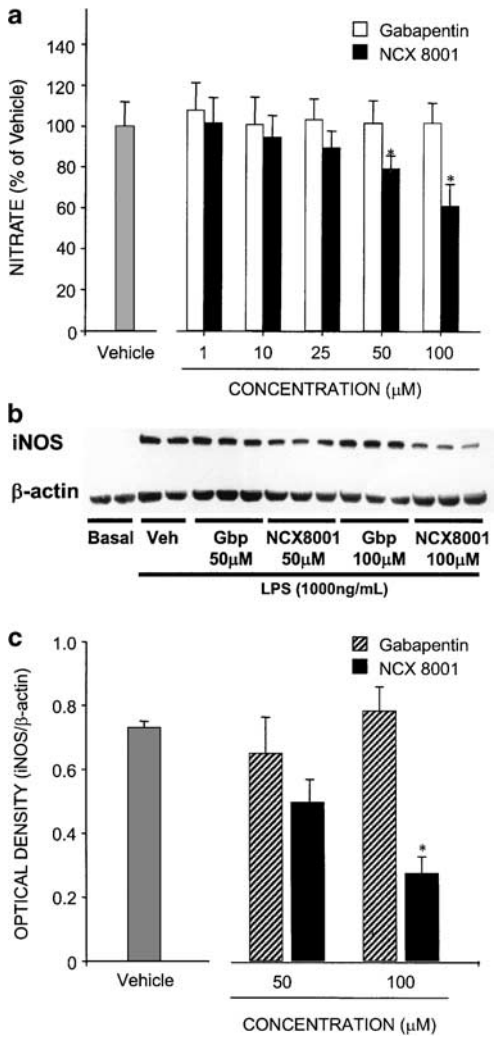

Figure 4 Effects of vehicle, gabapentin (Gbp) or NCX8001 on LPS-induced nitrate accumulation (a). Representative Western blot of iNOS and β-actin proteins following the exposure of RAW264.7 cells to 1000 ng ml^{-1} of LPS in presence of either vehicle, gabapentin or NCX8001 (b) and their respective quantitative analysis (c). Results from Western blot experiments are expressed as ratio between optical readings of iNOS and β-actin specific bands of at least three different determinations. $^*P<0.05$ as compared to vehicle or the respective concentration of gabapentin.

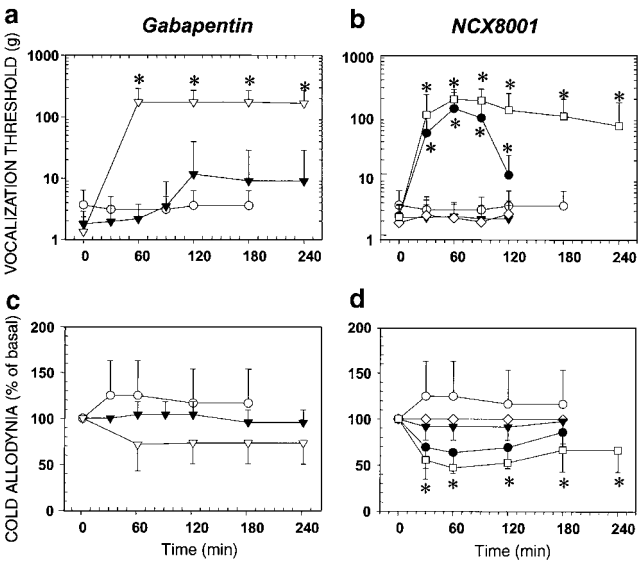

Figure 5 Effects of treatments on vocalization threshold to stimulation with von Frey hairs in spinally injured rats (a and b). Responses to cold stimulation are depicted in panel (c and d). Treatments were as follows: vehicle (open circles, $n=6$), gabapentin at 170 (closed triangles, $n=12$) and 580 μmol kg^{-1}, i.p. (open triangles, $n=10$); NCX8001 at 28 (closed triangles, $n=6$), 56 (open diamantes, $n=8$) 170 (closed circles, $n=8$) and 280 μmol kg^{-1}, i.p. (open squares, $n=6$). All data are expressed as mean$\pm$s.e.m. $^*P<0.05$ compared to predrug value at time 0 with Wilcoxon signed-ranks test or paired t-test.

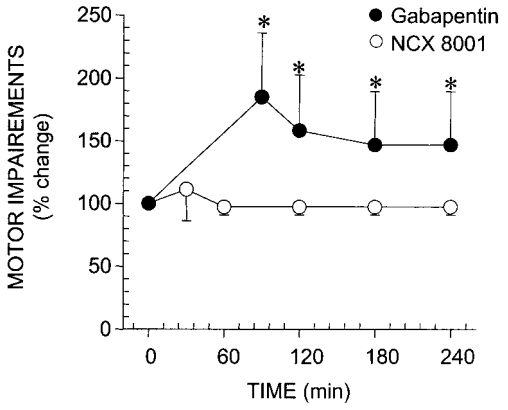

Figure 6 Effects on motor behavior following the administration of either a full effective antiallodynic dose (580 μmol kg^{-1}, i.p.) of gabapentin or the respective equipotent antiallodynic dose (280 μmol kg^{-1}, i.p.) of NCX8001 to spinally injured rats. Data are presented as percentage changes from the motor score (see also Methods) assigned to each animals prior to the administration of the drugs. $^*P<0.05$ compared to predrug value at time 0 with Wilcoxon signed-ranks test.

Administration of gabapentin to the allodynic rats up to 170 μmol kg^{-1}, i.p. (30 mg kg^{-1}), did not significantly alleviate the pain-like response of these animals to light, otherwise innocuous, mechanical and cold stimuli. The antiallodynic effects of gabapentin became evident when the drug was administered at higher dosage. In fact, the administration of 580 μmol kg^{-1}, ip, (100 mg kg^{-1}) significantly increased the vocalization threshold to von Frey hair stimulation and reduced cold allodynia (Figure 5a and c).

It is worth noting, however that this relatively high dose of compound also resulted in pronounced motor impairment and sedation that was not seen at lower dosages (Figure 6).

As shown in Figure 5b, the antiallodynic effects of NCX8001 was significant at 170 μmol kg^{-1}, i.p. (60 mg kg^{-1}) while the dose of 280 μmol kg^{-1}, i.p. (100 mg kg^{-1}) elicited a response that was equally effective and long-lasting (up to 360 min) to that observed after the highest dose of gabapentin (580 μmol kg^{-1}). Both the magnitude and duration of the effect were dose-related (Figure 5). Furthermore, NCX 8001, at doses that elicited an equipotent antiallodynic response to that of gabapentin, did not cause motor impairment or sedation (Figure 6).

As expected, the vehicle did not produce significant alteration of the baseline measurements.

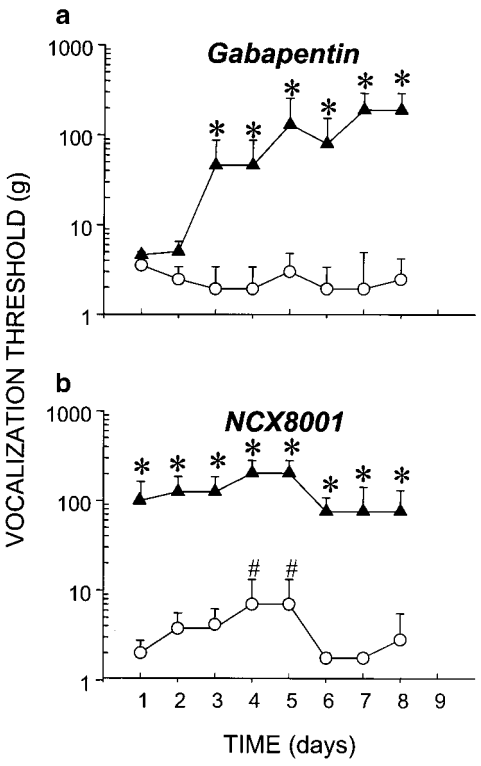

Figure 7 Effects of daily treatment with gabapentin (a, $n=9$) or NCX 8001 at 170 μmol kg^{-1}, i.p. (b, $n=6$) on vocalization threshold to von Frey hair stimulation in spinally injured rats. The vocalization threshold before (open circles) and 1 h after drug administration (filled triangles) are shown. $^{\#}P<0.05$ compared to day 1 with Wilcoxon signed-ranks test. $^{*}P<0.05$ compared to respective predrug value, Wilcoxon signed-ranks test or paired t-test.

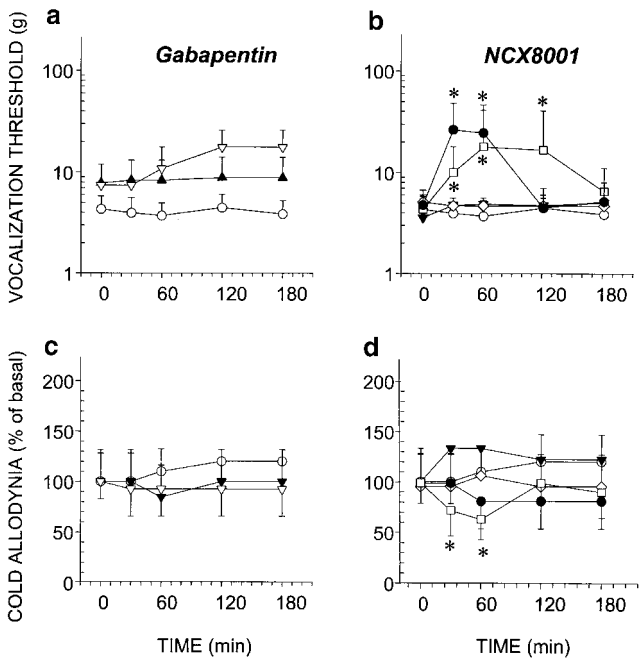

Figure 8 Effects of drug treatment on paw withdrawal threshold stimulation in peripheral nerve-injured rats (a and b). Responses to cold stimulation are depicted in panels (c and d). Treatments were as follows: vehicle (open circles, $n=6$), gabapentin at 170 (closed triangles, $n=12$) and 580 μmol kg^{-1}, i.p. (open triangles, $n=10$); NCX8001 at 28 (closed triangles, $n=6$), 56 (open diamantes, $n=8$) 170 (closed circles, $n=8$) and 280 μmol kg^{-1}, i.p. (open squares, $n=6$). All data are expressed as mean $\pm$ s.e.m. $^{*}P<0.05$ compared to predrug value (time 0) with Wilcoxon signed-ranks test.

Finally, the effects of equimolar doses of NCX8001 and gabapentin (170 μmol kg^{-1}) on mechanical allodynia were studied with a 8-day repeated administration schedule (Figure 7). Likewise in previous studies (Hao *et al*., 2000), gabapentin produced a significant effect starting at day 3 and the treatment did not affect baseline vocalization threshold.

NCX8001 induced antiallodynic effect from day 1 up to day 8 (Figure 7b). At the dosage used, neither drug elicited relevant motor dysfunction. Interestingly, there was also a temporary increase in baseline vocalization threshold following repeated NCX8001 treatment, which was significant at days 4 and 5.

Peripheral nerve injury

In rats with peripheral nerve injury, gabapentin did not significantly reduce any components of the pain-like behavior up to 580 μmol kg^{-1}, i.p. (Figure 8a and c) despite causing sedation and motor impairments at high dosage (data not shown).

In contrast, NCX8001 (28–280 μmol kg^{-1}, i.p.) dose-dependently alleviated mechanical and cold allodynia (Figure 8b and d) without causing major motor impairments and sedation.

Discussion

There is evidence that the antiepileptic drug, gabapentin, is effective in the clinical management of pain in different types of neuropathies (Backonja *et al*., 1998; Backonja & Glanzman, 2003). However, the use of gabapentin in therapy is hampered by its weaknesses ranging from the limited efficacy to side-effects liability. Thus, the medical need in this area remains high since alternative treatment options are limited.

With this background, we sought to investigate whether combining gabapentin to that of a NO-releasing moiety could result in a more effective drug. Therefore, we synthesized a series of derivatives bearing a NO-releasing group chemically bound through an aromatic bridge to the backbone of gabapentin. This approach has already been used with other drug classes to assure the concomitant presence of the parent drug and bioactive concentrations of NO slowly released in relevant biological tissues (Paul-Clark *et al*., 2000; Fiorucci *et al*., 2002).

Here, we report our findings on the lead compound, NCX8001, with respect to its ability to release NO *in vitro* as well as to its efficacy at ameliorating neuropathic pain-like behavior in two relevant animal models, namely, the central photochemical lesion of the spinal cord and the peripheral lesion of the sciatic nerve.

As expected, NCX8001 modulates the NO/cGMP signaling pathway as it increases cGMP content and induces vasorelaxant effects in NA-precontracted aortic rings. While NCX8001 efficiently counteracted the effects of NA, it only elicited marginal cGMP accumulation in PC12 cells unless the compound was applied in the presence of the NO-independent stimulator of sGC, YC-1. This discrepancy is not surprising as it has previously been reported that the potency of NO at stimulating sGC varies considerably in different systems

(Condorelli & George, 2001; Bellamy & Garthwaite, 2002). Various compounds have been shown to stimulate the activation of endothelial nitric oxide synthase (eNOS) and to promote the *de novo* synthesis and release of NO in biological tissues (Fulton *et al*., 1999). Hence, one might speculate that endogenous release of NO could have contributed to the different profile of NCX8001 in the two systems used. This possibility remains unlikely as we found that the potency and efficacy of NCX8001 at eliciting vascular relaxation was retained virtually unchanged in the absence of functional endothelium, which is the primary source of NO in this biological assay. Alternatively, as the release of NO from organic nitrates requires a series of enzymatic reactions, as yet not completely characterized, differences in specificity, cellular content and/or distribution of the enzymes involved in this metabolic processing could have accounted for the above discrepancy.

The major finding of this work is, however, that NCX8001 by virtue of its NO-releasing properties, results superior to gabapentin in alleviating neuropathic pain-like behaviors when administered to neuropathic rats. This is shown by both the enhanced potency of NCX8001 over gabapentin and the improved side-effect profile of this drug.

The mechanisms by which NCX8001 produced better analgesia compared to gabapentin are not entirely clear.

Given that NCX8001 is expected to form gabapentin *in vivo*, one possibility is that NCX8001 might have a more favorable pharmacokinetic/pharmacodynamic profile as compared to its parent counterpart. However, this possibility is not supported by preliminary experiments where the extent of gabapentin accumulation in the blood stream as well as at the spinal cord level did not differ significantly in animals treated with equimolar doses of the two drugs. Furthermore, the lack of sedation and motor impairments also suggest that the better profile of NCX8001 does not depend on higher amount of gabapentin delivered by this drug to the site of action. Nevertheless, at the present time, we cannot rule out this possibility.

Despite that gabapentin has proven effective, the mechanism/s of action of this drug are far from clear. However, recent evidence suggests that among other potential mechanisms, the effects of this drug may be partially mediated through the NO/cGMP signaling pathway (Dixit & Bhargava, 2002). This is not surprising given the well-recognized involvement of this pathway in the analgesic effect of several drugs (Duarte & Ferreira, 1992; Chiari *et al*., 2000; Jain *et al*., 2001; Lazaro-Ibanez *et al*., 2001). This makes it possible that the concomitant activation of the cGMP signaling pathway by both NO and gabapentin itself is responsible for the better antiallodynic profile of NCX8001 as compared to that of gabapentin alone.

The repeated administration of submaximal dose of NCX8001 resulted in an increased threshold to mechanical stimulation soon after the first injection and it remained stable for several days thereafter. Conversely, in line with previous reports (Hao *et al*., 2000) the repeated administration of gabapentin at doses that were subeffective when given acutely, elicited a significant analgesic response after three days. It is important to note, however, that repeated NCX8001 treatment also induced a temporary reduction in baseline allodynia, suggesting a possible cumulative effect of the drug leading to reversal of pain baseline. Alternatively, it cannot be excluded that with this treatment schedule, NCX8001 but not gabapentin counteracts the neuroplastic changes responsible of the enhanced excitability of spinal cord neurons.

It is becoming increasingly clear that neuropathic pain is associated with inflammatory reaction in the periphery and spinal cord (Millan, 1999). In this respect, particularly important is the paradoxal modulation that NO can exert on the expression and function of iNOS as well as of other proinflammatory mediators (Perez-Sala *et al*., 1999; Fiorucci *et al*., 2002). It is possible that NCX8001, by virtue of its NO-releasing properties, might have partially counteracted the inflammatory component generally ascribed to the release of proinflammatory mediators from locally recruited immune reactive cells and that this effect, in turn, contributed to the overall better profile of this drug following repeated treatment schedule (Watkins *et al*., 2001). Consistent with this idea, we found that prolonged exposure to NCX8001 but not gabapentin counteracted LPS-induced iNOS protein expression and function as well as of the proinflammatory cytokine, TNFα, in immortalized murine macrophages suggesting that this drug is endowed of anti-inflammatory properties. This effect is likely to depend on the NO-moiety as it was previously shown that other NO-releasing drugs also inhibited LPS-elicited proinflammatory cytokines accumulation and iNOS expression (Fiorucci *et al*., 2002). As to whether NCX8001 also exerts similar effects *in vivo*, remains to be established. It should be mentioned, however, that NCX8001 was also effective in a more classical model of peripheral inflammatory pain such as the mouse formalin test (data not shown) further supporting the concept that NCX8001 might exert its action, at least in part, through the modulation of the inflammatory pathway.

Overall, these results show that the addition of a NO-releasing moiety to gabapentin, a drug commonly used for the therapeutic management of neuropathic pain, leads to a more potent and better tolerated compound. Although the mechanism whereby NO acts has not been completely established, it is likely to involve the modulation of the inflammatory pathway.

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