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Title page

Repeated dosing with NCX1404, a nitric oxide (NO)-donating pregabalin, re-establishes normal nociceptive responses in streptozotocin (STZ)-induced painful diabetic neuropathy in mice

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Running Title Page

Running title: NCX1404 reduces mechanical allodynia development in STZ mice

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Abstract

NCX1404 is a novel nitric oxide (NO)-donating pregabalin readily absorbed and processed *in vivo* to pregabalin and NO. We determined the anti-allodynic response of NCX1404 following acute or after 7, 14 and 21 days of repeated daily oral dosing in streptozotocin (STZ)-induced painful diabetic neuropathy (PDN) in mice. Pregabalin and its combination with the NO donor, isosorbide mononitrate (ISMN) were used for comparison. Plasma levels of pregabalin and nitrites, used as surrogate marker of NO release, after NCX1404 or pregabalin dosing were monitored in parallel experiments using LC-MS/MS. NCX1404 and pregabalin resulted in similar pregabalin levels as it was their anti-allodynic activity after acute dosing in STZ mice. However, NCX1404 resulted in disease-modifying properties when administered daily for 21 days as indicated by time-and dose-dependent reversal of STZ-induced mechanical allodynia (PWT_{Veh_21d}=1.3 ± 0.15g for vehicle, $PWT_{NCX1404\_21d}$= 1.4±0.5g, 2.9±0.2g*, and 4.1±0.2g*, respectively 19, 63, and 190µmoles/kg, po of NCX1404, * p<0.05 vs. vehicle). This effect was not shared by pregabalin at equimolar doses (190µmoles/kg, po, PWT_{Pregab_21d}=1.4±0.1g, * p<0.05 vs. equimolar NCX1404). In addition, the NO donor, ISMN (52.3µmoles/kg, po) alone or combined with pregabalin (63µmoles/kg) was active at 7 days (PWT_{Veh_7d}=1.7±0.16g, PWT_{ISMN_7d}=3.9±0.34g*, PWT_{Pregab_7d}=1.3±0.07g and $PWT_{ISMN+pregab\_7d}$=3.8±0.29g*) but not at later time-points. The long-term effect of NCX1404 was independent from residual drug exposure and lasted for several days after the treatment was stopped. In summary, like pregabalin, NCX1404 is an effective anti-allodynic agent. Differently from pregabalin, repeated dosing of NCX1404 re-established normal nociceptive responses in STZ-induced PDN in mice.

Introduction

Neuropathic pain is a form of chronic pain that can be classified as peripheral or central. Peripheral neuropathic pain is caused by injury or infection of peripheral sensory nerves, whereas central neuropathic pain is caused by damage to the central nervous system (CNS) or/and the spinal cord. Both peripheral and central neuropathic pain can occur without obvious initial nerve damage. Various metabolic diseases cause neuropathic changes which later lead to neuropathic pain. An example is painful diabetic neuropathy (PDN) which occurs in a large number of patients suffering from diabetes. The pathophysiology of PDN remains unclear. Nevertheless, neurogenic and vascular components have been described (Yorek, 2003). For instance, peripheral demyelination, reduction in nerve conductance, and progressive degeneration of peripheral sensory fibers (Watkins, 1984) along with micro- and macro-vascular abnormalities dependent on dysfunctional endothelium, the innermost layer of the blood vessel, have long been established in experimental animal models and in diabetic patients (Pieper and Gross, 1988; Pitei et al., 1997) as well as in individuals who will later develop diabetes (Cosentino and Luscher, 1998).

To date, the antidepressant duloxetine (King et al., 2015) and the anticonvulsants gabapentin and pregabalin (Schreiber et al., 2015) are considered the first-line treatment strategies for patients affected by various forms of neuropathic pain including PDN. These drugs are generally safe, but they do not entirely relieve pain symptoms and a substantial subset of patients is virtually insensitive to treatment. Furthermore, even when they work, these and other available treatments ameliorate the symptoms but lack effects on the underlying pathophysiology of the disease, making clinical management difficult as the disease progresses to its late stages.

Depending on the circulating concentration and the specific tissue compartment where it is released, nitric oxide (NO) exerts various functions ultimately leading to either pro-nociceptive or anti-nociceptive behaviors (Miclescu and Gordh, 2009; Schmidtko et al., 2009). Low physiologic amounts of NO activate soluble guanylyl cyclase (sGC) causing vascular relaxation, favor neuronal transmission, and modulate genes transcription through nuclear factor (NF)-kB signaling (Colasanti and Suzuki, 2000), whilst large quantities of NO are thought to produce abnormal neuronal activity and central sensitization (Schmidtko et al., 2009), activate pro-inflammatory cytokines, and promote tissue-specific oxidative and nitrosative stress, which also are featured in diabetic conditions (Pacher et al., 2007). Different laboratories (Sasaki et al., 1998; Rodella et al., 2000) have demonstrated that impaired NO generation in diabetic rats results in hyperalgesia while the administration of L-arginine, known to enhance the circulating NO concentration, is antinociceptive in diabetic mice. Pitei and co-workers showed that decreased NO production contributes to reduced endoneuronal blood flow in diabetic patients and caused pain (Pitei et al., 1997), while the local application of NO donors, such as isosorbide dinitrate (Yuen et al., 2002) or nitroglycerin (Francis et al., 1977; Agrawal et al., 2007), relives pain and burning sensation in patients with PDN.

Early exploratory work from our laboratories documented the ability of NO to enhance the effect of gabapentin in neuropathic pain models (Wu et al., 2004). Additional evidence of NO-mediated effects in neuropathic pain models was later obtained with compounds possessing both antioxidant and NO-releasing properties (Ronchetti et al., 2009). Other investigators reported the beneficial effect of NO when combined with gabapentin in spite of the different experimental conditions employed (Curros-Criado and Herrero, 2009). Similarly, Huang and co-workers, documented the analgesic effects of an NO-donating

derivative in a rat model of PDN (Huang et al., 2015), while Otari and Upasani reported on the involvement of NO-cGMP signaling in neuropathic pain processing (Otari and Upasani, 2015).

In the present study, we investigated the anti-allodynic effects of a novel NO-donating pregabalin derivative, NCX1404, following a single treatment schedule. Furthermore we addressed whether repeated daily dosing with NCX1404 modulates the development and maintenance of mechanical allodynia consequent to STZ. In all experiments, the NO donor isosorbide 5-mononitrate (ISMN) and its combination with pregabalin were used for comparison.

Materials and Methods

In all experiments, animals were cared for and treated in accordance with protocols approved by the Institutional Animal Care Committees, and all efforts were made to limit the number of animals and to minimize animal suffering. Male CD1 mice, weighting 20-30 g were kept under standard conditions of temperature (22-24°C) and illumination (12:12-h light/darkness). They were allowed to adjust to this environment in cages with mesh bottoms with free access to fresh water and food for at least 7 days before the experiments began.

NCX1404 ((3S)-5-methyl-3-(((1-(4-(nitrooxy)butanoyloxy)ethoxy)carbonylamino) methyl)hexanoic acid) is a nitrooxyderivative of pregabalin synthesized in our laboratories in 8 steps with 12% overall yield. The compound was obtained by coupling pregabalin and 1-{[(4-nitrophenoxy)carbonyl]oxy}ethyl 4-(nitrooxy)butanoate (9) that was prepared in 7 steps from 4-bromobutanoic acid (Figure 1). To obtain the corresponding 15N- labeled NCX1404, 15N-$AgNO_3$ was used instead of $AgNO_3$ in step b reported in Figure 1.

15N-Isosorbide 5-mononitrate was synthesized in house by adding 15N-Bu_4NNO_3 , 2,6-di-tert-butyl-4- methyl pyridine and trifluoromethanesulfonic anhydride to a solution of isosorbide in dichloromethane. 15N-Isosorbide 5-mononitrate was then isolated by flash chromatography.

Other chemicals and reagents were purchased from Sigma-Aldrich, St Louis, MO unless otherwise specified.

Streptozotocin (STZ)-induced diabetes mice model

Diabetes was induced by injecting 200 mg/kg i.p. of streptozotocin (STZ) (Kowaluk et al., 2000; Vincenzi et al., 2014). Blood glucose levels were assessed from the second day until the end of the experiment using an Accu-Chek blood glucose monitoring system. A single administration of STZ induced insulin-dependent diabetes mellitus within 24-48 h by destruction of pancreatic islet cells (Schenkman, 1991). Plasma glucose levels above 300 mg/dl were considered indicative of diabetes (Migita et al., 2009).

Mechanical allodynia paw withdrawal test

Paw withdrawal thresholds (PWT) were determined using the Dynamic Plantar Aesthesiometer (Ugo Basile, Italy), an apparatus that generates a mechanical force linearly increasing with time (Szolcsanyi et al., 2004; Wright et al., 2007). Mice were placed individually in plastic cages with a wire mesh bottom and allowed to acclimatize for at least 2 h. Increasing mechanical stimulation (0.25 g/s, cut-off force: 10 g) was applied to the plantar surface of a hind paw. The nociceptive threshold was defined as the force, in grams, at which the mouse withdraws its paw. When a withdrawal response occurred, the stimulus was terminated and the response threshold measured electronically. Data are reported as the mean ± standard error of the threshold recorded at each trial.

Drug treatment paradigm

Acute schedule. On day 14 post STZ challenge, mice were treated orally with a single challenge of drugs at the indicated doses or vehicle (Hypromellose 0.5%, DMSO 2%). Mechanical allodynia, expressed as paw withdrawal threshold (g) was evaluated at 0, 5, 30, 45, 60, 90, 120 and 180 min after treatments.

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***Repeated treatment schedule*.** On day 7 post-STZ challenge, mice were treated orally each day with the selected drugs at the indicated doses or vehicle (Hypromellose 0.5%, DMSO 2%) for 21 days. Mechanical allodynia expressed as paw withdrawal threshold (g), was measured on day 7, 14 and 21, 18-24 h after the last daily treatment. In addition, changes in the mechanical threshold were continued to be monitored in all groups at 3, 7 and 10 days after dosing was discontinued.

Analytical assessments

Parallel experiments were conducted to monitor the plasma levels of NCX1404 and pregabalin after acute administration of NCX1404 or pregabalin at 63 μmole/kg, po. For the acute schedule experiments, blood samples were collected from the left ventricle prior to (0) and at 5, 15, 30, 60, 120, 240 and 360 min post-dose in anesthetized mice (n=3 for each time point). The blood samples were later processed for further plasma isolation and analytical assessments. 15N-Nitrites, used as surrogate marker of NO release, were monitored at the same time points as above in animals treated with 15N-NCX1404 at the same dose (63 μmole/kg, po). For the repeated dosing experiments, blood samples were withdrawn 18-24h after 7, 14, or 21 days of consecutive daily treatment with 63 μmole/kg, po of pregabalin or NCX1404 and later processed to monitor the levels of pregabalin in plasma.

NCX1404 and pregabalin quantitation. Aliquots (50 μL) of plasma were added with 10 μL of 100% DMSO, 10 μL H_2O, and 500 μL methanol and then centrifuged at 4000 rpm for 10 min, 4°C. The supernatants were collected and stored at -80°C and later analyzed by a sensitive and specific LC/MS/MS method for simultaneous determination of pregabalin and NCX 1404. The system was an Acquity UPLC (Waters, Milford, MA). The detector was a

Quattro microTM API LC/MS/MS (Waters, Milford, MA). The conditions used to analyze NCX1404 and pregabalin were: Column, ACQUITY BEH Phenyl 50 x 2.1 mm (5 µm) at 40°C; Mobile Phase, A. water + 0.1% formic acid and B. methanol + 0.1% formic acid; flow rate 0.5 mL/min; Detector Wavelength, 210 nm; MRM transitions (Multiple Reaction Monitoring) in ESI positive for NCX 1404 was m/z 400.92 → 325.07; for pregabalin was m/z 160.2 → 97.1. The raw data (average peak area at each time point) were interpolated using Quanlynx v4.1 software to obtain the concentration of compounds at each time point.

15N-Nitrites. Blood samples were taken from the left ventricle prior to (0) and at 5, 15, 30, 60, 120, 240 and 360 min post-dose, the plasma separated and later extracted with acetonitrile. The quantification is based on conversion of 15N-Nitrites to 15N-naphtotriazole using 1.2-diaminonaphtalene in acidic conditions, followed by quantification by LC-MS/MS.

Briefly, the system used was an Agilent 1200 HPLC (Agilent Technologies, Santaclara, CA). The detector was an API4000 mass spectrometer (Applied Biosystems). 15N-Nitrites conversion to naphtotriazole was done mixing 200 µL of deproteinized blood sample spiked with 10µL of 1.2-diaminonaphtalene 1mM in HCl 0.5 M and shaken for 20min at room temperature. At the end, to stop the conversion, 10 µL of NaOH 1M and 10µL of DMSO were added to all the samples. The resulting compound was then separated from the mixture using an ACE C18 AR 50 x 2.0 mm (3 µm) column. Mobile phase: A) water/0.1% formic acid and B) acetonitrile/0.1% formic acid. MRM transition (Multiple Reaction Monitoring) in ESI positive for 15N-naphtotriazole was m/z 171.1 → 115.0. Raw data (average peak area at each time point) were interpolated on a standard curve to obtain the concentration of 15N-naphtotriazole at each time point.

Statistics

In all experiments, comparison between treatments groups was performed by two-way ANOVA followed by a Bonferroni multiple comparison test unless otherwise specified. P values below 0.05 were considered significant.

Results

Blood glucose levels and mechanical allodynic threshold following streptozotocin (STZ)-treatment in CD-1 mice

Physiologic glucose plasma levels in CD-1 mice were 149 ± 4 mg/dl. The administration of streptozotocin (STZ) resulted in a rapid increase in plasma glucose levels starting from the following day, and the levels remained elevated throughout the weeks thereafter reaching 515 ± 17 mg/dl at 4 weeks post-treatment (Table 1).

The injection of STZ also significantly diminished the paw withdrawal threshold (PWT) to mechanical stimulation starting 14 days after STZ injection (PWT, 4.2 ± 0.31 and 1.3 ± 0.44 g, vehicle and STZ, respectively). The allodynic response remained stable over the following 2 weeks (Table 1).

NCX1404, pregabalin plasma levels after NCX1404 and pregabalin in mice

The plasma levels of NCX1404 in mice after 63 μmole/kg, po (23,7 mg/kg) were below the limit of quantitation at all time-points tested. NCX1404 is a nitrooxy ester derivative of pregabalin, we thus compared the extent of circulating pregabalin as well as that of nitrites (serving as surrogate marker of NO release) after NCX1404 (63 μmoles/kg, po) or equimolar pregabalin (63 μmoles/kg, po) single dosing. Parallel experiments were made using NCX1404 labelled on the nitrate group with 15N to study the levels of nitrites released into the blood stream after NCX1404 single dosing. Pregabalin levels after NCX1404 single dosing reached the C_{max} of 6.8±0.6 μg/mL 30 min post NCX1404 administration and declined in the next hours to reach levels close to baseline after 360

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min (Figure 2A). Pregabalin administered under similar conditions resulted in similar pregabalin exposure compared to that found after NCX1404 (Figure 2A). The relative pregabalin bioavailability after NCX1404 compared to pregabalin was 85%.

15N-Nitrites plasma levels after NCX1404 and isosorbide 5-mononitrate in mice.

15N-Nitrites plasma levels after NCX1404 oral treatment (63 μmole/kg, po) were maximal 5 min after dosing the animals and rapidly declined to basal levels thereafter (Figure 2B). Conversely, 15N-Nitrites after isosorbide 5-mononitrate (ISMN) oral dosing (52.3 μmole/kg, po) in the plasma increased slowly to reach maximum at 60 min (C_{max}=1.77±0.4μM) and slowly decayed thereafter to reach basal values during the following 4 h (Figure 2B).

Anti-allodynic effects of acute dosing of NCX1404 or pregabalin in streptozotocin (STZ)-induced painful diabetic neuropathy mice model

As shown in figure 3A, acute administration of NCX1404 at 19, 63, and 190 μmoles/kg, po, (7.1, 23.7, and 71 mg/kg) reduced mechanical allodynia in a time- and dose-dependent fashion ($PWT_{NCX1404\_60min}$=2.6±0.2g, 3.9±0.3g, and 4.6±0.2g for 19, 63, and 190 μmoles/kg, po of NCX1404, respectively) vs vehicle ($PWT_{Vehicle\_60min}$=1.3 ± 0.2g) in STZ-induced painful diabetic neuropathy (PDN) mice model. The administration of pregabalin at equimolar doses (3, 10 and 30 mg/kg) also reduced mechanical allodynia in a time- and dose-dependent fashion (PWT_{Pregab_60min}=2.8±0.1g, 3.8±0.3g, 4.3±0.4g after 19, 63 and 190 μmoles/kg, po of pregabalin, respectively) reaching values close to those

observed in naïve animals (Figure 3B). Consistent with pregabalin exposure after NCX1404 or pregabalin oral treatment, the anti-allodynic responses of both drugs were maximal between 45 and 60 min post-dosing and slowly disappeared thereafter to reach basal values at 120 min (Figure 3A and B).

Reversal of the allodynic response following 21-day repeated dosing with NCX 1404 in streptozotocin (STZ)-induced painful diabetic neuropathy mice model

These experiments were undertaken to address whether repeated daily administration of NCX1404 or pregabalin re-establish normal nociceptive responses to mechanical stimulation in STZ-induced PDN mice model. The compounds were administered by oral gavage starting 1 week post-STZ treatment whereas testing was performed at 7, 14 and 21 days, 18-24 h after the respective daily treatment to assure the absence of circulating pregabalin which was then confirmed in parallel pharmacokinetic experiments by the absence of detectable levels of pregabalin in plasma samples collected at the same time-points (data not shown). Furthermore, the duration of the effect was monitored by assessing the mechanical threshold at 1, 3, 7 and 10 days after the 21 days of repeated daily dosing was ended. Similar treatment paradigm with pregabalin at equimolar doses was used for comparison.

The repeated oral administration of 19 μmoles/kg (7.1 mg/kg), 63 μmoles/kg (23.7 mg/kg) and 190 μmoles/kg (71 mg/kg) of NCX1404 resulted in dose- and time-dependent enhancement of mechanical threshold which was maximal after 21 days (Figure 4A). The effect of NCX1404 was retained for several days after ending the daily treatment as indicated by the high mechanical threshold still recorded 3 and 7 days after the treatment

was completed (Figure 4A). Regardless of the dose used, this effect was not shared by pregabalin administered at equimolar doses under the same experimental conditions (Figure 4B) suggesting that these effects likely depend on the release of NO.

Anti-allodynic response following acute or 21-day repeated dosing with the NO donor, isosorbide 5-mononitrate, pregabalin or their combination in streptozotocin (STZ)-induced painful diabetic neuropathy mice model

We tested whether isosorbide 5-mononitrate (ISMN) *per se* or combined with pregabalin exerts any anti-allodynic effect on STZ-induced PDN mice model. In exploratory experiments, ISMN was tested at 15.7 μmoles/kg, po (3 mg/kg), 52.3 μmoles/kg, po (10 mg/kg) and 157 μmoles/kg, po (30 mg/kg). In these experiments, 15.7 μmoles/kg did not modify the allodynic threshold to mechanical stimulation in STZ-treated animals whereas the high dose of 157 μmoles/kg resulted in significant anti-allodynic activity combined with robust cardiovascular hypotension (data not shown). Conversely, the administration of ISMN at 52.3 μmoles/kg resulted in significant anti-allodynic effects with only moderate hypotension, transient in nature and thus, this dose was selected for further experiments. Animals treated with 52.3 μmoles/kg, po of ISMN showed mild anti-allodynic response as indicated by the increase in PWT at selected time-points post dosing (Figure 5). Likewise, 63 μmoles/kg, po (10 mg/kg) administration of pregabalin also elicited mild to moderate anti-allodynic effects as in previous experiments (Figure 5). However, the concomitant oral administration of ISMN (52.3 μmoles/kg, po) and pregabalin (63 μmoles/kg, po) resulted as marginally effective as either drug given alone.

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To address whether STZ-induced mechanical allodynia was influenced by ISMN, we then monitored the allodynic response after 7, 14 and 21 days of daily oral treatment with ISMN (52.3 μmoles/kg, po) in animals kept treatment-free for 18-24 h prior to testing so to assure the absence of circulating NO during testing. In these experimental sessions, pregabalin (63 μmoles/kg, po) alone or combined with ISMN (52.3 μmoles/kg, po) administered once/day for 21 consecutive days were used for comparison.

Repeated daily dosing with 52.3 μmoles/kg, po (10 mg/kg) of ISMN reduced the development of mechanical allodynia in mice treated with STZ one week earlier. In particular, the effect was evident after 7 days of repeated daily treatment but disappeared during the following days (Figure 6). Similarly transient was the effect recorded after the co-administration of pregabalin (63 μmoles/kg, po) and ISMN (52.3 μmoles/kg, po) (Figure 6).

Discussion

NCX1404 is a dual acting nitric oxide (NO)-donating derivative of pregabalin featuring a mononitrate alkyl chain bound to the amino function of pregabalin by an acyloxy-alkyl carbamate. This novel compound is designed to combine the NO-mediated anti-inflammatory and anti-oxidant properties with those of pregabalin, which is known to reduce the symptoms of pain while lacking any measurable effect on the underlining pathophysiology.

The administration of streptozotocin (STZ) to mice results in sustained diabetes (Kowaluk et al., 2000) and measurable allodynic response to mechanical stimulation. These effects are thought to be consequent to the progressive degeneration of peripheral sensory fibers (Watkins, 1984) due to dysfunctional vascular endothelium and reduced NO availability (Yorek, 2003). The STZ model is thus particularly interesting to address the acute anti-allodynic effects as well as long-term changes of the nociceptive circuits associated with the administration of NO-donating drugs like NCX1404.

In our hands, mice receiving a single challenge of STZ rapidly increased their plasma glucose level and later developed robust allodynic response to mechanical stimulation which was maximal within 7 days and remained stable over several weeks thereafter. For this reason, day 14 post STZ treatment was selected to test the acute anti-allodynic response of NCX1404 versus pregabalin while 7 days post-STZ was used as starting point for repeated dosing in order to address the effects of NCX1404 on the development of allodynia in diabetic mice.

NCX1404 is readily absorbed following oral dosing in STZ mice and efficiently cleaved in vivo to release pregabalin as for other molecules carrying similar approach (Cundy et al.,

2004). In addition, NCX1404 also releases relevant amounts of NO as determined by the presence of 15N-nitrites in plasma of animals dosed orally with 15N-labelled NCX1404. The anti-allodynic effect of NCX1404 after a single oral challenge in STZ-induced diabetic neuropathic mice model was time- and dose-dependent. Similarly, equimolar doses of pregabalin also resulted in time- and dose-dependent antiallodynic effects that were overlapping with that elicited by the respective equimolar dose of NCX1404. Furthermore, the antiallodynic effects of both NCX1404 and pregabalin after single acute oral dosing were coincident with the respective time-course of pregabalin plasma exposure. These data indicate that the acute anti-allodynic effects of NCX1404 are mostly dependent on pregabalin release with minor, if any, contribution of NO. Alternatively, the absence of superiority maybe due to partially overlapping mechanisms for pregabalin and NO. In a different series of experiments, the single oral administration of the NO-donor, isosorbide 5-mononitrate (ISMN) resulted in modest anti-allodynic effect after mechanical stimulation which was similar to that of a sub-maximal dose (10mg/kg) of pregabalin. Moreover, their co-administration (concomitant pregabalin and ISMN dosing) was only as effective as the individual compounds given alone consistent with the concept that the acute anti-allodynic effect of NO and pregabalin could be due, at least partially, to overlapping mechanisms. Pregabalin mainly alleviates neuropathic pain symptoms by impairing the trafficking of α2δ-1 receptors and, subsequently, reducing Ca^{2+} influx and spinal sensitization (Sills, 2006). However, additional mechanisms involving the activation of NO/cGMP signaling pathway have recently been described for another α2δ-1 antagonist, gabapentin (Mixcoatl-Zecuatl et al., 2006; Takasu et al., 2006). Whether similar mechanisms also take place for pregabalin remain to be established.

The main observation made in the present study is, however, the significant time- and dose-dependent long-term changes of the nociceptive circuits exhibited by NCX1404 after repeated daily dosing in mice with established neuropathy consequent to the administration of STZ 1-week earlier. The enhancement of nociceptive threshold following NCX1404 long-term dosing reflect changes in the nociceptive circuits rather than direct anti-allodynic effects of the drug and its active metabolites as no detectable levels of pregabalin and NO were measured at the time (18-24h after the last daily dose) the behavioral assessments were taken. This effect appears to be unique to NO-donating drugs like NCX1404, as pregabalin, administered under the same experimental conditions, did not result in measurable activity. Similar trend, albeit of different intensity, was observed when the NO-donating derivative of gabapentin (NCX 1236) was administered under the same conditions (supplemental Figure 1) suggesting the importance of NO release in mediating this effect. Moreover, ISMN administered alone or combined with pregabalin was as effective as NCX1404 on day 7 but not on day 14 and 21, time at which ISMN-mediated tolerance occurs (Daiber and Munzel, 2015). These latter results further indicate the importance of NO release in the allodynic responses elicited by STZ after repeated dosing with NCX1404 and, at the same time, suggest potential differences between NCX1404 and ISMN with respect to their tolerance liability, ability to efficiently release NO over repeated dosing and/or efficiency to activate the NO/cGMP signaling pathway (Klemenska and Beresewicz, 2009). NCX1404 releases pregabalin and the nitrate linker *via* the action of esterases (data not shown); however, we still lack information on how the nitrate linker, once cleaved from pregabalin, releases NO. In our experiments, the kinetics of 15N-nitrites, used as a surrogate marker of NO release, differed significantly after 15N-NCX1404 and 15N-ISMN dosing. In spite of similar C_{max}

between the two compounds, 15N-nitrites accumulation was rapid (T_{max} 5min) and decayed quickly after 15N-NCX1404. Conversely, 15N-nitrites measured after 15N-ISMN were largely superior compared to that from 15N-NCX1404 with a T_{max} at 60min. Furthermore, the bioactivation and NO release from ISMN occur via the P450 enzyme that is down-regulated when repeatedly stimulated (Minamiyama et al., 2004). This mechanism, along with soluble guanylyl cyclase desensitization and activation of oxidative stress have been described to mediate ISMN-induced tolerance (Minamiyama et al., 2004; Daiber and Munzel, 2015) but this mechanism may not be as important for NCX1404 as similar NO-donating compounds were reported to rely on different bioactivation pathways compared to ISMN (Govoni et al., 2006).

The effect of NCX1404 is independent from residual drug as pregabalin levels in plasma samples withdrawn at the time of behavioral assessments were found below the limit of quantitation (data not shown). Further to that, the reduction of mechanical allodynia in animals treated with NCX1404 for 21 days was still evident 3 and 7 days after the treatment was ended making it unlikely that residual compound accounts for the observed activity. NCX1404 did not affect plasma glycemic levels (data not shown) in these animals explaining why, soon after the treatment was completed, the allodynic threshold returned to levels similar to that of vehicle with a time-course resembling that observed after the initial injection of STZ.

The molecular and cellular mechanisms underlying the long-term effects observed after NCX1404 repeated dosing are far from clear. Neurogenic and vascular changes are involved in the pathophysiology of PDN (Yorek, 2003). These include the release of various inflammatory mediators (Purwata, 2011), vascular endothelial cell dysfunction, and progressive reduction of NO which is thought to play a pivotal role in the pathophysiology

of PDN (Pieper and Gross, 1988). NCX1404 releases biologically relevant amounts of NO as detected by the plasma increase in 15N-nitrites. Thus, the circulating NO brought about by NCX1404 might compensate for the reduced endogenous NO production consequent to endothelial cell dysfunction in STZ-treated animals. Likewise, circulating NO from NCX1404 might inhibit inflammatory cytokines release. Consistent with this interpretation, previous studies documented the ability of NO donors to ameliorate endothelial function in rodent models of diabetes (Pieper and Gross, 1988; Ambrosini et al., 2005; Huang et al., 2015) and to reduce inflammatory cytokines via modulation of NF-kB translocation (Colasanti and Suzuki, 2000; Ronchetti et al., 2006).

In summary, acute dosing of NCX1404 is as effective as acute dosing of pregabalin to reverse mechanical allodynia in STZ-diabetic mice. However, NCX1404, differently from pregabalin, when administered over 21 days re-establishes normal nociceptive responses in STZ-induced PDN in mice. This result is likely to be due to a mechanism involving the release of NO.

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Authorship contributions

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Participated to research design: Katia Varani and Francesco Impagnatiello

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Footnotes

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Figure legends

Figure 1. Synthesis of NCX 1404.

a) $SOCl_2$, MeOH, r.t, overnight, 85% ; b) $AgNO_3$, CH_3CN, reflux, 4 hours 93 % ; c) LiOH 2 N, MeOH, 5 °C, overnight, 84%; d) Ag_2O, CH_3CN / H_2O, r.t., 3 hours, 70% ; e) 1-chloroethyl chloroformate, Py, CH_2Cl_2, r.t., overnight, 97% ; f) NaI, CH_3CN, 53% ; g) dry toluene, r.t., overnight, 71% ; h) pregabalin, triethylamine, trimethylsilylchloride, CH_2Cl_2, r.t., overnight.

Figure 2. A) Pregabalin exposure after single oral administration of NCX 1404 or pregabalin to mice. NCX 1404 (closed circle, n=3) and pregabalin (open circle, n=3) were administered at 63 μmole/kg, po, (23.7 and 10 mg/kg for NCX1404 and pregabalin, respectively) dissolved in vehicle (Hypromellose 0.5%, DMSO 2%). Both drugs were given by oral gavage at 5ml/kg. B) 15N-Nitrites were monitored following the administration of NCX1404 (63 μmol/kg, closed circle, n=3) labelled with 15N given at the same dose and conditions as above of ISMN (52.3 μmol/kg, open square, n=3) labelled with 15N.

Figure 3. Dose-dependent anti-allodynic effect of NCX 1404 (A) and pregabalin (B) in STZ-diabetic mice. Both drugs were administered dissolved in vehicle (Hypromellose 0.5%, DMSO 2%, grey triangle, n=6). NCX1404 was given by oral gavage (5ml/kg) at 19 μmole/kg (7.1mg/kg, closed diamond, n=6), 63 μmole/kg (23.7mg/kg, closed circle, n=6) and 190 μmole/kg (71 mg/kg, closed square, n=6). Pregabalin was administered at equimolar doses as NCX1404 (3 mg/kg, open diamond, n=6; 10 mg/kg, open circle, n=6; 30 mg/kg, open square, n=6). Paw withdrawal threshold following mechanical stimulation allodynia was monitored at 0 (basal), 15, 30, 45, 60, 90, 120 and 180 min post treatment.

*p<0.05 vs vehicle, Two way ANOVA followed by Bonferroni's multiple comparisons test (NCX 1404 19 μmole/kg at 45 min; NCX 1404 63 μmole/kg from 30 to 90 min; NCX 1404 190 μmole/kg from 15 to 90 min; pregabalin 19 μmole/kg from 45 to 60 min; pregabalin 63 μmole/kg from 30 to 90 min; pregabalin 190 μmole/kg from 30 to 90 min).

Figure 4. Dose-dependent effects of repeated daily oral dosing with of NCX 1404 (A) or pregabalin (B) in STZ-diabetic mice. Both drugs were administered dissolved in vehicle (Hypromellose 0.5%, DMSO 2%, grey triangle, n=6). NCX1404 was given by oral gavage (5ml/kg) at 19 μmole/kg (7.1 mg/kg, closed diamond, n=6), 63 μmole/kg (23.7 mg/kg, closed circle, n=6) and 190 μmole/kg (71 mg/kg, closed square, n=6). Pregabalin was administered at equimolar doses as NCX1404 (3 mg/kg, open diamond, n=6; 10 mg/kg, open circle, n=6; 30 mg/kg, open square, n=6). All treatments were given daily between 9 and 10AM for 21 days starting 7 days after the initial STZ challenge. All measurements of mechanical allodynia were performed in drug-free conditions. The later aspect was verified by confirming the absence of a detectable concentration of pregabalin in plasma samples withdrawn at the time the experiment was performed. *p<0.05 vs vehicle, Two way ANOVA followed by Bonferroni's multiple comparisons test (NCX 1404 63 μmole/kg from 7 to 21 days; NCX 1404 190 μmole/kg from 7 to 28 days).

Figure 5. Acute anti-allodynic effect of ISMN alone or combined with pregabalin in STZ-diabetic mice. ISMN (52.3 μmole/kg, po equal to 10mg/kg, cross, n=6), pregabalin (63 μmole/kg, po equal to 10mg/kg, open circle, n=6) or their combination (closed triangle, n=6) were administered orally (5mL/kg) dissolved in vehicle (Hypromellose 0.5%, DMSO 2%, grey triangle, n=6). Mechanical allodynia was monitored at 0 (basal), 15, 30, 45, 60, 90 and 120 min post dosing. *p<0.05 vs vehicle, Two way ANOVA followed by Bonferroni's

multiple comparisons test (ISMN 52.3 μmole/kg from 15 to 45 min; pregabalin 63 μmole/kg from 30 to 45 min).

Figure 6. Repeated daily oral dosing with of ISMN, pregabalin or their combination in STZ-diabetic mice. ISMN (52.3 μmole/kg, po equal to 10mg/kg, cross, n=6), pregabalin (63 μmole/kg, po equal to 10mg/kg, open circle, n=6) or their combination (closed triangle, n=6) were administered daily dissolved in vehicle (Hypromellose 0.5%, DMSO 2%, grey triangle, n=6) between day 1 and day 21 starting 7 days after the STZ challenge. All measurements of mechanical allodynia were performed in drug-free conditions immediately before the treatment of the respective day. *$p<0.05$ vs vehicle, Two way ANOVA followed by Bonferroni's multiple comparisons test (ISMN 52.3 μmole/kg from 7 to 14 days; ISMN+pregabalin from 7 to 14 days).

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Table 1. Time course changes in blood glucose level (A) and mechanical allodynic threshold (B) in mice injected with streptozotocin (STZ)

Time (days)	**Blood glucose levels** mg/mL	**Paw withdrawal threshold** (g)	
	STZ-treated	**Vehicle**	**STZ-treated**
0	149.4 ± 4.3	4.1 ± 0.22	4.3 ± 0.21
2	386.5 ± 20.1*	----	----
7	476.2 ± 18.2*	4.0 ± 0.23	2.1 ± 0.26
14	496.0 ± 11.1*	4.2 ± 0.31	1.3 ± 0.44*
21	529.3 ± 12.7*	4.3 ± 0.22	1.2 ± 0.16*
28	514.6 ± 17.4*	4.4 ± 0.21	1.2 ± 0.33*

Streptozotocin (200 mg/kg i.p., n=10) or vehicle (distilled water, n=10) were administered by intraperitoneal injection at 5 mL/kg. The data are expressed as mean ± S.E.M. * $p < 0.05$ vs time 0 and vehicle at the respective time points.

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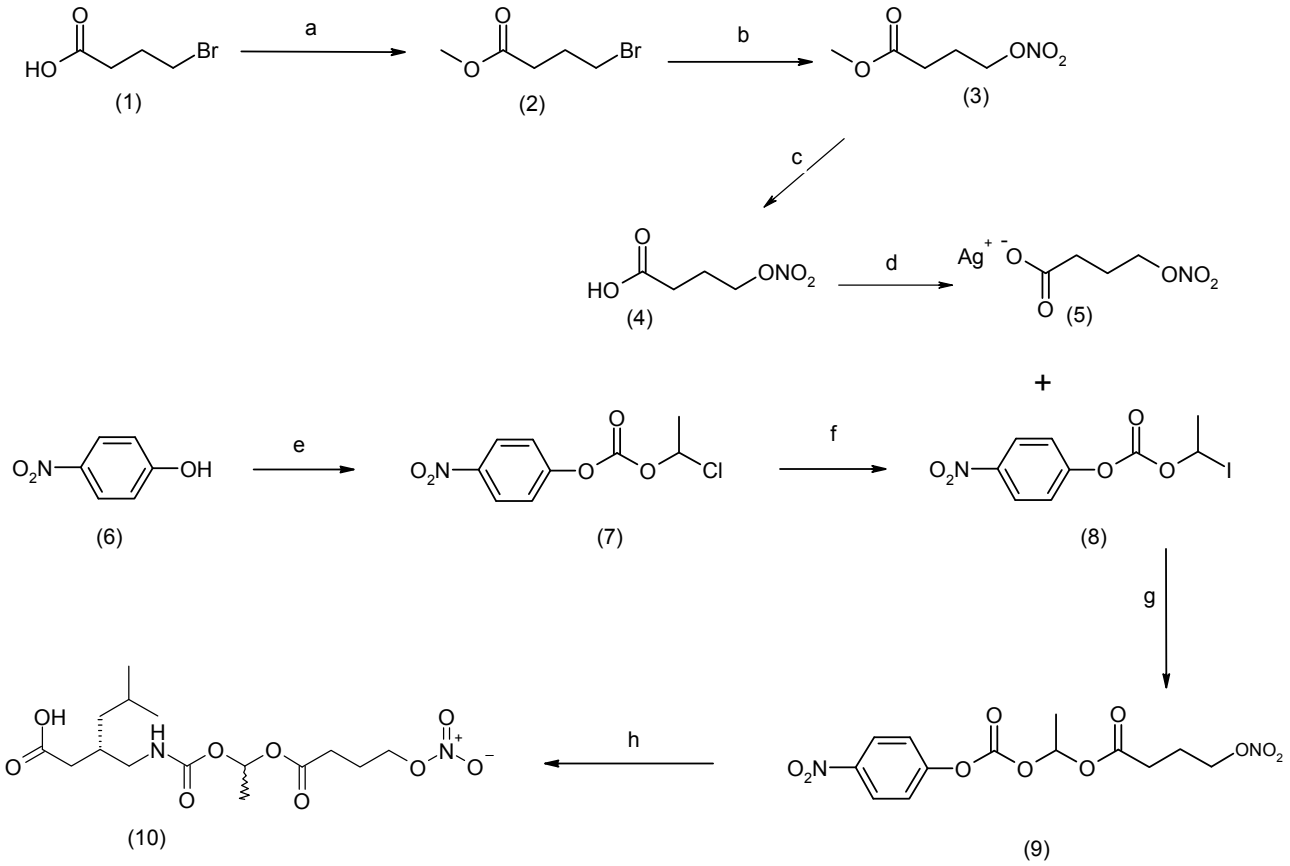

Figure 1

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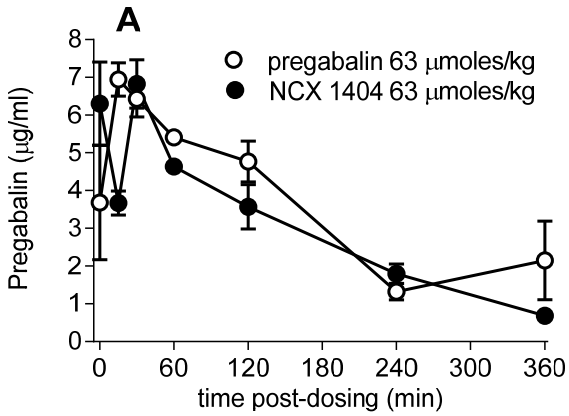

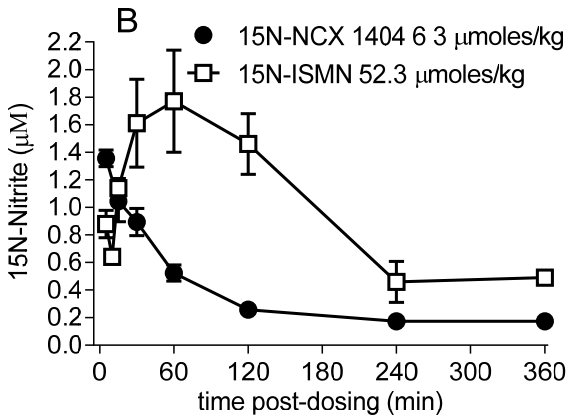

Figure 2

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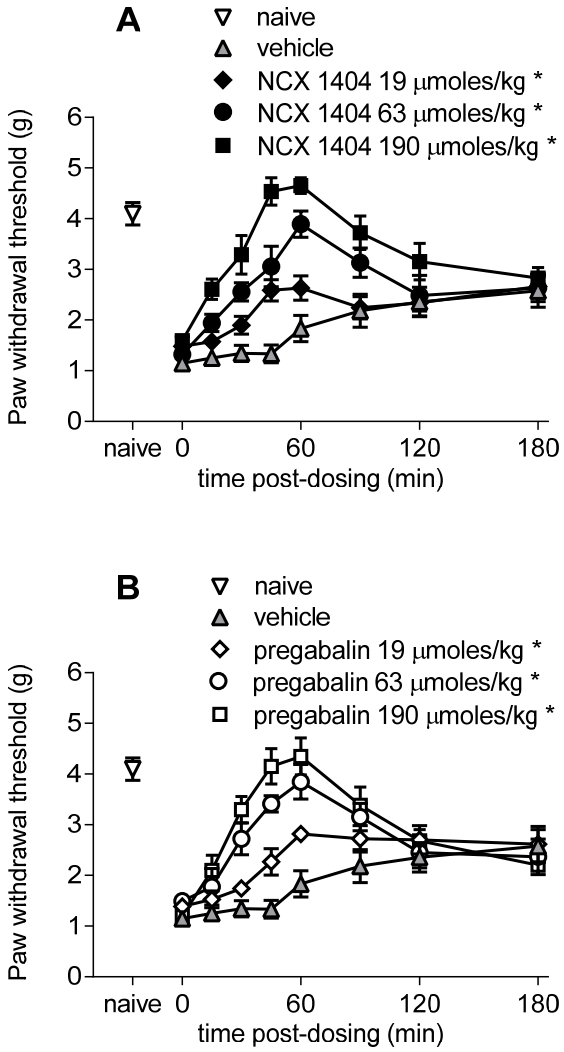

Figure 3

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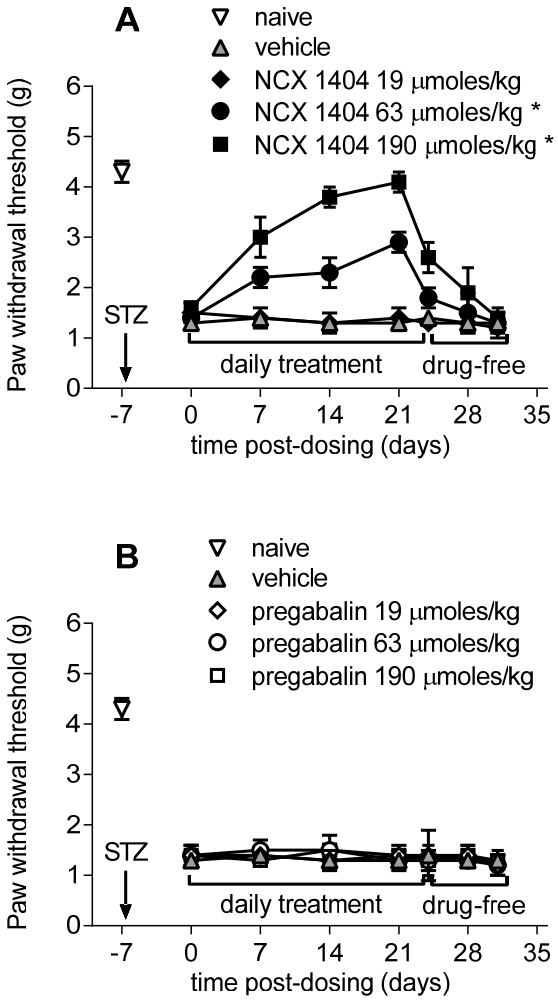

Figure 4

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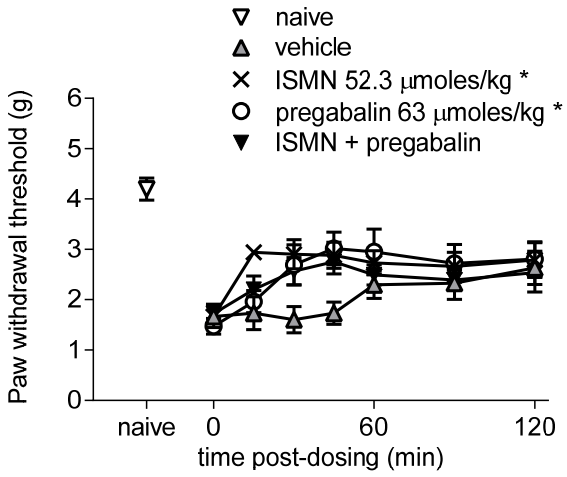

Figure 5

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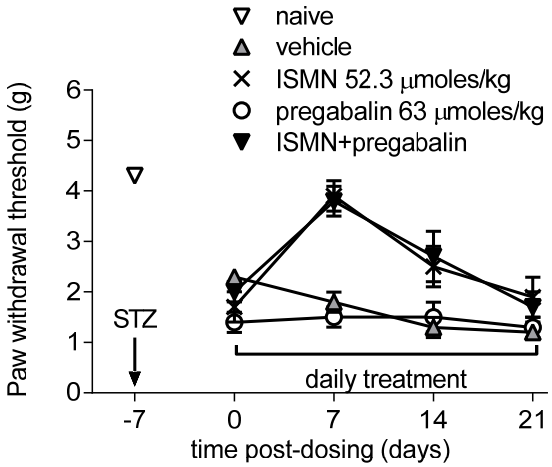

Figure 6