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Specific Anti-nociceptive Activity of Cholest-4-en-3-one, Oxime (TRO19622) in Experimental Models of Painful Diabetic and Chemotherapy-induced Neuropathy

Thierry Bordet, Bruno Buisson, Magali Michaud, Jean-Louis Abitbol, Fabien Marchand, John Grist, Emile Andriambeloson, Marzia Malcangio and Rebecca M. Pruss

Trophos, Parc Scientifique de Luminy, Luminy Biotech Entreprise, Case 931, 13288 Marseille, Cedex 9, France (T.B., B.B., M.M., J-L.A., R.M.P.); Neurorestoration Group Wolfson CARD, King's College London, London SE1 1UL, UK (F.M., J.G., M.M.); Neurofit, Parc d'Innovation, 67400 Illkirch, France (E.A.)

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Address correspondence to:

Dr Rebecca M. Pruss, Trophos, Parc Scientifique de Luminy, Luminy Biotech Entreprises, Case 931, 13288 Marseille Cedex 9, France. Tel +33 491 828 281; Fax +33 491 828 289; E-mail: rpruss@trophos.com

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4-MC, 4-Methylcatechol; ALCAR, acetyl-L-carnitine; ANOVA, analysis of variance; CCI, chronic constriction injury; CMAP, compound muscle action potential; mPTP, mitochondrial permeability transition pore; NGF, nerve growth factor; NT-3, neurotrophin-3; ROS, reactive oxygen species; SNCV, sensory nerve conduction velocity; STZ, streptozotocin; TRO19622, cholest-4-en-3-one, oxime

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ABSTRACT

Diabetes and cancer chemotherapies are often associated with painful neuropathy. The mechanisms underlying neuropathic pain remain poorly understood and the current therapies have limited efficacy and are associated with dose-limiting side effects. We recently described the pharmacological characterization of cholest-4-en-3-one, oxime (TRO19622), a cholesterol-like compound, which significantly reduced axonal degeneration and accelerated recovery of motor nerve conduction in a model of peripheral neuropathy induced by crushing the sciatic nerve. These results triggered investigation of efficacy in other preclinical models of peripheral neuropathy. Here we report evidence that daily oral administration of TRO19622, while similarly improving motor nerve conduction impaired in streptozotocin-induced diabetic rats, also reversed neuropathic pain behavior early as the first administration. Further exploration of these acute anti-nociceptive effects demonstrated that TRO19622 was also able to reverse tactile allodynia in vincristine-treated rats, a model of chemotherapy-induced neuropathic pain. Interestingly, TRO19622 did not have analgesic activity in animal models of pain produced by formalin injection, noxious thermal or mechanical stimulation or chronic constriction injury of the sciatic nerve, indicating that painful diabetic or chemotherapy-induced neuropathies share a common mechanism that is distinct from acute, inflammation or lesion-induced neuropathic pain. These results support the potential use of TRO19622 to treat painful diabetic and chemotherapy-induced neuropathies.

INTRODUCTION

Neuropathic pain is a puzzling and complex condition that may develop when nerve fibers are damaged or dysfunctional. Diabetes and some types of cancer chemotherapy result in peripheral neuropathy associated with loss of sensation and numbness in the feet, hands and legs accompanied by painful tingling or burning sensations (Visovsky, 2003; Said, 2007). Slowing in sensory nerve conduction, nociceptor hyperexcitability and changes in central pathways that interpret and modulate sensory transmission are found in diverse syndromes besides diabetic and chemotherapy-induced neuropathy such as post-herpetic neuralgia, acquired immunodeficiency syndrome therapy-induced neuropathy and nerve injuries. Changes in ion channel expression may account for these perturbations (Luo et al., 2002; Hong et al., 2004; Amir et al., 2006). In the clinic, anti-convulsants, anesthetics, antidepressants and opiates are used to treat neuropathic pain although there is a clear medical need for new treatments to improve both efficacy and safety (Dworkin et al., 2007).

Trauma, inflammation, diabetes and chemical insults (alcohol, human immunodeficiency virus anti-retroviral therapies and cancer chemotherapeutics) produce allodynia and hyperalgesia in response to mechanical stimuli in animal models. However, the molecular mechanisms underlying neuropathic pain differ in these models (Ahlgren et al., 1997; Siau and Bennett, 2006; Chen and Levine, 2007). Chronically elevated blood glucose in diabetes is associated with increased production of reactive oxygen species (ROS),decreased nerve blood flow, reduced supply of trophic factors, slowed nerve conduction and evidence of damaged mitochondria; if not corrected, these defects lead to degenerative abnormalities in axonal nerve fibers (reviewed by Vincent et al., 2004; Leinninger et al., 2006; Tomlinson and Gardiner, 2008). Chemotherapy with platins, taxanes or vinca alkaloids induces neuropathic

pain behavior in rats that mimicks that in seen in patients (Cata et al., 2006). While vincristine and paclitaxel inhibit tumor cell proliferation by disrupting microtubule polymerization, neuropathic pain behavior appears even in the absence of profound microtubule depolymerization and axonal loss. Although partial degeneration of intraepidermal receptor sensory nerve arbors was evidenced in rats treated with vincristine or paclitaxel (Siau et al., 2006) pain behavior was rather correlated with the presence of vacuolated mitochondria in peripheral nerves (Flatters and Bennett, 2006).

We recently described the identification and pharmacological characterization of TRO19622 (cholest-4-en-3-one, oxime), a compound that binds to two outer mitochondrial membrane proteins and has remarkable neuroprotective properties (Bordet et al., 2007). In a model of peripheral neuropathy induced by crushing the sciatic nerve, TRO19622 treatment reduced axonal degeneration and accelerated recovery of motor nerve conduction. These findings led to the assessment of the effects of TRO19622 in three models of painful peripheral neuropathy streptozotocin (STZ)-induced diabetes, cancer chemotherapy (vincristine) and chronic constriction nerve injury (CCI). The selective anti-nociceptive effects of TRO19622 shown in this paper provide experimental evidence for a novel approach to treatment of debilitating pain associated with diabetic or chemotherapy-induced peripheral neuropathies.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Janvier (Le Genest Saint Isle, France). Male Wistar rats were obtained from Harlan (UK) or from BioLasco (Taiwan). Rats were housed under temperature-controlled (21-24°C) and 12h/12h light-dark cycles with free access to food and water. All animals were cared for and pain studies performed in accordance with the European Communities Council Directive (86/609/EEC) and with the ethical guidelines of the Committee for Research and Ethical Issues of IASP (Zimmermann, 1983). Protocols were approved by institutional veterinary ethics committees.

Induction of diabetes

Diabetes was induced in Sprague Dawley (175-200 g) or Wistar (~250 g) rats by a single intravenous or intraperitoneal injection of STZ (Sigma, 55 mg/kg). Animals showing blood glucose levels above 260 mg/dl (15 mmoles/L) approximately 1 week after STZ injection were considered hyperglycemic and diabetic.

Induction of neuropathic pain with vincristine

Vincristine sulphate (Sigma) was dissolved in 0.9% NaCl and Wistar rats (~250 g) were injected with 200 μg/kg intravenously (1 mL/kg) on days 1, 4 and 6 (total cumulative dose 600 μg/kg).

Test compounds

TRO19622 was synthesized by Archemis (Décines Charpieu, France) or Synkem (Chenôve, France). To prepare solutions for oral administration, the compound was ground to a fine

powder and mixed with the required quantity of vehicle (corn oil) to obtain the concentrations corresponding to the dose level to be administered in a volume of 5 mL/kg. Oral treatment with TRO19622 by gavage was conducted once a day in the morning.

4-Methylcatechol (4-MC, Sigma) was dissolved in 0.9% NaCl and vortexed until complete dissolution. Intraperitoneal administration with 10 µg/kg 4-MC was performed daily beginning with a dosage volume of 1 mL/kg.

Morphine (except as noted below) was dissolved in 0.9% NaCl and vortexed until complete dissolution and administered subcutaneously 45 minutes prior to behavioral testing at a dose of 3 mg/kg in a volume of 1 mL/kg.

Gabapentin (except as noted below), synthesized by American Custom Chemicals (San Diego, USA), was freshly prepared in 0.9% NaCl before use and administered orally (50 mg/kg, 5 mL/kg) twice a day.

Nerve conduction studies

Sensory and motor nerve conduction in diabetic rats was assessed prior to (day -3) and on days 8, 25 and 40 post-STZ injection using electrophysiological measurements, as previously described in this model (Andriambeloson et al., 2006). Rats were anaesthetized by intraperitoneal injection of 60 mg/kg ketamine chlorhydrate (Imalgene 500®, Rhône Mérieux, Lyon, France). Normal body temperature was carefully maintained with a heating lamp and monitored by a contact thermometer (Quick, Bioblock Scientific, Illkirch, France) placed on the tail surface. Electrophysiological recordings were performed using a Neuromatic 2000M electromyograph (Dantec, Les Ulis, France). Sensory nerve conduction velocity (SNCV) was

recorded from the tail of the rat using a reference needle inserted at the base of the tail and an anode needle placed 30 mm away from the reference needle towards the extremity of the tail with a ground needle electrode inserted between the anode and reference needles. The caudal nerve was stimulated with a series of 20 pulses (for 0.2 ms) at an intensity of 12.8 mA and SNCV was expressed as m/s. The compound muscle action potential (CMAP) was recorded in the gastrocnemius muscle after stimulation of the sciatic nerve. A reference electrode and an active needle were placed in the hind paw. A ground needle was inserted on the lower back of the rat. The sciatic nerve was stimulated with a single 0.2 ms pulse at supramaximal intensity. The latency of the motor wave was recorded and expressed in ms. Daily administration of 4-MC, a potent stimulator of nerve growth factor (NGF) synthesis (Hanaoka et al., 1992), was used as a positive control in these studies.

Behavioral testing in diabetic and vincristine-treated rats

All experiments were performed 'blind' in a quiet room by a single experimenter using the method of equal blocks with randomization of treatments in order to avoid any uncontrollable environmental influence that might introduce variability in behavioral responses. Baseline responses were recorded in each animal before surgery, STZ or vincristine injection.

Assessment of thermal allodynia and mechanical hyperalgesia in STZ-injected rats

Thermal allodynia was assessed using a warm (38°C) plate as described previously in this model (Andriambeloson et al., 2006). Briefly, animals were placed into a glass cylinder located on a hot plate adjusted to 38°C. The latency of the first reaction was recorded (licking, moving the paws, little leaps or a jump to escape the heat) with a cutoff time of 30 s.

Mechanical hyperalgesia was assessed with the Randall and Sellito test using a paw pressure analgesimeter (Bioseb, France) which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. The mechanical nociceptive threshold was defined as the force in grams at which the rat withdrew its paw. The cut off pressure was set to 250 g. Testing was performed 4h after the administration of TRO19622, 4-MC or vehicle (corn oil) or 45 min after injection of morphine.

Assessment of tactile allodynia in STZ- and vincristine-injected rats

Tactile allodynia was measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Briefly, each animal was placed in a clear acrylic cubicle (22 x 16.5 x 14 cm) with a metal grid floor giving access to the underside of their paws and allowed to acclimate for 15 min before testing. A mechanical stimulus was applied to the plantar surface of one hind paw by a stainless steel filament (0.5mm diameter) exerting a linearly increasing force (2.5g/s). The force (g) at which paw withdrawal occurred was automatically recorded. Each rat paw withdrawal threshold was calculated as the average of three consecutive tests performed at 5 min intervals by an experimenter blind to the treatments. A cut-off of 50g was imposed to prevent tissue damage. Animals injected with STZ were tested once a week from week 3 to 5 and those displaying significant allodynia on week 5 compared to their baseline response were randomly assigned to groups for testing effects of TRO19622, vehicle (corn oil) or gabapentin. Testing was performed on five consecutive days 4h after administration of TRO19622 or vehicle and 3h after administration of the morning dose of gabapentin.

Assessment of tactile allodynia induced by sciatic nerve ligation

The CCI procedure was performed on male Wistar rats (160-200 g) as initially described (Bennett and Xie, 1988). After anesthesia with pentobarbital (50 mg/kg, i.p.), the sciatic nerve was exposed at mid-thigh level and three ligatures (4-0 silk suture) about 1mm apart were loosely tied around the nerve. The animals were housed individually in cages with soft bedding for 7 days before testing for tactile allodynia. On the first test day, rats were placed under inverted Plexiglas cages on a wire mesh rack and allowed to acclimate for 20 minutes. Responsiveness to a #12 Supertip® (IITC, USA) applied beneath the mesh floor perpendicular to the central plantar surface of the hind paw was assessed on both the lesioned and non-lesioned side. A positive response to the applied tactile pressure, noted by a sharp withdrawal of the paw was recorded automatically by an Electronic Von Frey Aesthesiometer (2390CE Electrovonfrey®, IITC, USA). Response was the mean of two consecutive measures separated by 1.5 min. Rats were selected for clear presence of allodynia if the response measured on the operated side 7 days after nerve ligation was reduced by at least 10 g of force relative to the response prior to nerve ligation. Test compounds were administered once a day for 5 consecutive days by oral gavage to groups of 5 animals and testing for tactile allodynia was performed 1h after gabapentin (Pfizer; 200 mg/kg in 2% Tween 80; 10 mL/kg) or 4h after vehicle (corn oil) or TRO19622 (3, 30 or 300 mg/kg). While this dose of gabapentin is higher than that used in the studies with diabetic or vincristine-treated rats (above) testing is performed earlier, at a time when plasma levels are likely to be similar based on published pharmacokinetic data (Cundy et al., 2004).

Behavioral response to subplantar formalin injection

Male Wistar rats (~130 g) were fasted overnight prior to use. TRO19622, 300 mg/kg, and morphine (60 mg/kg; 10 mL/kg) were administered by oral gavage to groups of 6 animals 4h or 1h, respectively, before subplantar injection of formalin (0.05 mL, 5% solution) in one hind

paw. Hind paw licking time (s) was measured at 5-minute intervals for 30 min after formalin injection. Values from 0-5 min and 15-30 min were regarded respectively as the early phase and late phase of the pain response.

Assessment of TRO19622 plasma concentrations

TRO19622 was analyzed in blood samples obtained from 3-6 animals per group ~4 hours following the last administration. The blood was collected in lithium-heparin tubes then centrifuged at 1200 rpm to obtain plasma, which was stored frozen until analysis by HPLC-MS/MS.

Statistical analysis

Results were expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis was assessed by ANOVA followed by Student-Newman-Keuls post test with p values less than 5% deemed significant.

RESULTS

Effects of TRO19622 on nerve conduction and pain behavior in diabetic rats

By one week following a single injection of STZ, blood glucose levels in diabetic rats were 4-5 times higher compared to saline-treated rats (544 ± 24 mg/dL in STZ rats versus 117 ± 3 mg/dL in controls) and remained high until the last measurement on day 40 in a study of the effects of TRO19622 on diabetes-induced nerve conduction deficits that also included exploratory assessment of pain behavior. As expected, body weight stagnated in STZ-injected rats while it increased steadily in control rats (from 248 ± 6 g to 456 ±26 g in controls versus 247 ± 8 g to 289 ± 50 g in STZ rats, respectively, day 0 and day 40). Daily treatment with TRO19622 or 4-MC had no effect on hyperglycemia or body weight of STZ-injected rats (Table 1).

Similar to the effects seen following a crush injury to the sciatic nerve, TRO19622 treatment from day 10 to day 40 significantly reduced CMAP latency in diabetic rats. By day 40 an improvement in CMAP latency of 10-65% was seen over the dose range tested (3-300 mg/kg/d) compared to vehicle treated rats and treatment with the positive control, 4-MC, had a similar effect on CMAP latency as the 30 mg/kg/d dose of TRO19622 (Figure 1A). Despite these effects on motor nerve conduction, neither 4-MC nor TRO19622 treatment significantly altered SNCV dysfunction in diabetic rats by day 40 (Figure 1B).

Exploratory studies of the effect of TRO19622 on pain behavior were performed on two occasions during the nerve conduction study. First, the acute effects of TRO19622 on the thermal allodynia 10 days following STZ injection was assessed 4h following a single oral administration. Second, the effects of prolonged treatment (ten daily doses) of TRO19622 or

4-MC on mechanical hyperalgesia 20 days after STZ injection were evaluated. By 10 days post-STZ, diabetic rats had already developed thermal allodynia based on the average response time and the number of animals that responded prior to the 30 s cut-off both in the 38°C hot plate test (Table 2) and in the cold bath test (data not shown). Interestingly and unexpectedly, there was a trend for the 30 and 300 mg/kg dose of TRO19622 to reverse STZ-thermal allodynia 4h following a single oral administration although the 3 mg/kg dose was inactive. On day 20 post-STZ, mechanical hyperalgesia measured using a Randall-Sellito analgesimeter demonstrated a marked and significant decrease in the paw withdrawal latency in diabetic rats as compared to control animals (79.2 ± 5.5 g in STZ-vehicle rats *versus* 175.8 ± 10 in controls; $p < 0.001$; Table 2). While repeated treatment with 3 mg/kg TRO19622 from day 10 had no effect, both the 30 or 300 mg/kg doses significantly reversed paw withdrawal latency compared to vehicle treated diabetic rats (113.5 ± 7.7 g and 110.4 ± 9.1 g, respectively; $p < 0.05$) and these effects were similar to those obtained with morphine (131.2 ± 6.8 g; $p < 0.001$). It should be noted that despite its similar beneficial effects on motor nerve conduction, 4-MC treatment had no effect on pain behavior of diabetic rats in the paw pressure test (80.4 ± 8.9 g; $p > 0.05$). As there was no difference in glycemia or body weight between groups of STZ-injected rats in this study (see above), the anti-nociceptive effects of TRO19622 cannot be attributed to a general improvement in the health of the animals.

TRO19622 reverses tactile allodynia in chronically diabetic rats

To further assess anti-allodynic effects of TRO19622 in diabetic rats, a second study was specifically designed to measure its ability to reverse tactile allodynia, a clinically relevant behavior. As previously described STZ-induced diabetes produced a significant decrease in the threshold to mechanical stimulation that is fully developed five weeks after STZ injection ($p < 0.05$, Figure 2). Remarkably, 4h after a single oral administration, TRO19622 reversed

diabetes-induced mechanical allodynia in a dose-related manner that was statistically significant with the highest dose (100 mg/kg; Figure 2A). Repeated administration of TRO19622 for five consecutive days produced significant anti-allodynic effects at 10 and 30 mg/kg on days 2, 4 and 5 and at 100 mg/kg on days 1, 2 and 5 compared to vehicle treated rats ($p < 0.05$, Figure 2B) similar to the effects of gabapentin, 50 mg/kg twice a day.

TRO19622 reverses tactile allodynia in a rat model of chemotherapy-induced neuropathic pain

The anti-nociceptive effect of TRO19622 was next evaluated in a rat model of vincristine-induced neuropathic pain. Rats developed tactile allodynia within two days following the first injection of vincristine (Figure 3; day 3, $p = 0.015$), which was fully established after the third injection (day 7, $p < 0.001$) and remained significant up to 8 days after the last injection of vincristine in vehicle-treated animals (day 7-14, $p < 0.001$). As in diabetic rats, TRO19622 significantly reversed vincristine-induced allodynia 4h after the first oral administration of the highest dose tested (100 mg/kg, day 10, $p < 0.001$; Figure 3A). Although lower doses did not produce a significant anti-allodynic effect on the first day of administration repeated treatment with 10, 30 and 100 mg/kg all significantly reversed vincristine–induced allodynia from day 11 to day 14 compared to vehicle treated animals (Figure 3B). Gabapentin (50 mg/kg, twice a day) also effectively reversed tactile allodynia on days 10 - 14.

TRO19622 has no effect on pain behavior either in normal animals or in a CCI model of neuropathic pain

To further explore the effects of TRO19622 in models of neuropathic pain in rats, the response to treatment with 3, 30 and 300 mg/kg/d was studied on five consecutive days starting 7 days following surgery to produce a chronic constriction injury to the sciatic nerve. In this study neither acute nor chronic treatment with TRO19622 reversed tactile allodynia measured in the lesioned hind paw while gabapentin (200 mg/kg/d) fully reversed allodynia on all days (Figure 4A). In this study, potential sedative or anesthetic effects of TRO19622 were ruled out since there was no change in responses measured in the non-lesioned hind paw even at the highest dose of 300 mg/kg (Figure 4B). Likewise, TRO19622 (100 mg/kg) had no effect on paw withdrawal thresholds of normal rats in tests of mechanical allodynia or mechanical hyperalgesia (Figure 5). TRO19622 was also inactive in the formalin test; a single oral administration of 300 mg/kg TRO19622 4h prior to subplantar injection of formalin had no significant effect on either the first or second phase of the pain response while morphine (60 mg/kg) was efficacious in both phases of this model (Table 3).

Relationship between TRO19622 plasma concentration and anti-nociceptive efficacy

Plasma TRO19622 concentrations were determined at the end of each study (following the final testing session) approximately 4h after the last administration of TRO19622 (Table 4). Except for the formalin test, in which blood sampling was performed after a single administration of 300 mg/kg, all other values reflect the steady-state concentration of TRO19622 after repeated administration. Previous pharmacokinetic studies in rats showed that TRO19622 has an elimination half-life of approximately 24h leading to accumulation with steady state levels in plasma achieved after three daily oral administrations (data not shown), which accounts for the approximate 5-fold higher plasma concentration determined in the vincristine and CCI studies after 5 days repeated administration of 300 mg/kg

TRO19622 compared to that found 4h following a single administration in the formalin test. Interestingly, TRO19622 plasma concentrations in STZ-diabetic rats were about twice as high as those found in rats treated with vincristine or in the CCI study. These data can be used to estimate a dose and plasma concentration for TRO19622 to reverse neuropathic pain behavior. Since 3 mg/kg had no effect on pain behavior in diabetic rats (STZ nerve conduction study) even with repeated administration, a steady state plasma concentration of 0.8 µM appears to be below the level needed for efficacy. Likewise, a single administration of 10 mg/kg, which results in a similar plasma concentration (~0.7 µM; data from pharmacokinetic studies) did not significantly reverse pain behavior. However, repeated administration of 10 mg/kg significantly reversed pain behavior and resulted in steady state plasma concentrations of between 2 and 4.5 µM in vincristine-treated and diabetic rats (STZ pain study), respectively. A single dose of 100 mg/kg TRO19622 effectively reversed pain behavior after a single administration with plasma concentrations between 14.2 and 37.5 µM in the vincristine and STZ pain studies, respectively. Based on these data, we conclude that a minimal efficacious dose of TRO19622 for reversal of pain behavior is correlated with a plasma concentration at or above 2 µM, which can be achieved with a single administration of 100 mg/kg or repeated administrations of 10 mg/kg.

DISCUSSION

We report here the first description of the anti-nociceptive activity of TRO19622, a mitochondrial-targeted neuroprotective compound. Daily oral administration of the compound reversed both thermal and tactile allodynia in streptozotocin-induced diabetic rats. Similarly, TRO19622 reversed tactile allodynia in vincristine-treated rats, a model of chemotherapy-induced neuropathic pain. These effects were observed as early as the first administration and persisted after chronic administration. Interestingly, TRO19622 did not have analgesic activity in models of acute noxious pain or ones with inflammatory components such as formalin-induced pain or chronic constriction nerve injury model.

TRO19622 effects on diabetic peripheral neuropathy

We previously reported the identification of TRO19622 from a screening and chemical optimization program on rat primary motor neurons deprived of trophic factors (Bordet et al., 2007). TRO19622 promoted motor neuron survival and neurite outgrowth *in vitro*. In a model of peripheral neuropathy induced by crushing the sciatic nerve, TRO19622 treatment reduced axonal degeneration and accelerated recovery of motor nerve conduction. Here we assessed its effects in a model of peripheral neuropathy induced by diabetes monitoring both nerve dysfunction and the pain symptoms. Slowing of motor and sensory-nerve conduction is an early sign of neuronal dysfunction in diabetic rats and in patients. These early defects are directly linked to glucose neurotoxicity and are reversed by establishing normoglycaemia. Interestingly these deficits do not correlate with any structural impairment in axonal diameter or in myelin sheath and are rather due to altered ion fluxes and currents (Tomlinson and Gardiner, 2008). Here reduction in motor nerve conduction was evidenced as early as 8 days post-STZ injection in diabetic rats. Chronic treatment with TRO19622 from day 10 to day 40

improved motor nerve conduction up to 55% of the vehicle value. Interestingly, improvement in motor nerve conduction in STZ-injected rats could be detected with chronic oral administration of doses as low as 3 mg/kg/d producing steady state plasma concentrations of ~0.8 μM (Table 4), which is similar to that found with the lowest active dose of TRO19622 shown to accelerate nerve recovery in mice following a sciatic nerve crush (Bordet et al., 2007). In addition, TRO19622 did not modify hyperglycemia or increase body weight in STZ-injected rats excluding a general improvement in animals' condition as an explanation for its effects on motor nerve conduction. Our results rather suggest that TRO19622 restores motor nerve conduction either directly or indirectly. Here 4-MC, a potent stimulator of endogenous NGF synthesis, also reduced nerve impairment in diabetic rats as previously reported (Hanaoka et al., 1992; Hanaoka et al., 1994). As suggested by these findings, diabetic rats showed significant reduction in NGF and neurotrophin-3 (NT-3) in sciatic nerve and in innervated skin and muscle (Hellweg et al., 1994; Fernyhough et al., 1998). Interestingly, exogenous administration of both NGF and NT-3 reversed axonal transport and conduction deficits in STZ-diabetic rats (Fernyhough et al., 1995; Mizisin et al., 1999). Altogether these results suggest that impaired neurotrophic support is involved in the development of the diabetic neuropathic process. TRO19622, like 4-MC, may reverse the effect of STZ-diabetes on motor nerves by providing or compensating for loss of trophic support.

TRO19622 effects on neuropathic pain syndromes

STZ-induced diabetes in rats is associated with mechanical hyperalgesia and tactile allodynia (Courteix et al., 1993; Calcutt et al., 1996; Malcangio and Tomlinson, 1998). In two independent studies we found that TRO19622 reverses neuropathic pain behaviors in diabetic rats. Interestingly, TRO19622 was found to reverse both thermal and established tactile allodynia following the first oral administration with efficacy comparable to that of morphine

or gabapentin. This acute anti-nociceptive effect was dose dependent with a single administration and significant at the highest dose while lower doses exhibited similar efficacy after repeated administration. This suggests that accumulation allows lower doses of the compound to reach effective levels. After repeated administration, even at high dose, no habituation to the drug occurred. Interestingly, treatment with 4-MC produced a similar improvement in nerve conduction but had no effect on pain behavior in diabetic rats. Thus the beneficial effects of TRO19622 on pain behavior appear to be in addition to and not the result of improvement in nerve conduction. TRO19622 also reversed tactile allodynia produced by cumulative doses of vincristine implying a common mechanism underlying diabetic and chemotherapy-induced neuropathic pain. A direct effect of TRO19622 on ion channels involved in pain behavior appears to be ruled out on the basis of previous *in vitro* and *in vivo* observations (Bordet et al., 2007). TRO19622 also has no sedative or anti-convulsant activity even after repeated administration of up to 300 mg/kg/d for 5 days in mice (Supplemental data) and as reported here, did not demonstrate analgesic activity in pain models where anti-convulsants, opiates and local anaesthtics are active. At doses up to 300 mg/kg/d TRO19622 had no effect on tactile allodynia in a model of CCI-induced neuropathic pain and did not reverse either the acute or late phases of the formalin test. TRO19622 also had no effect on normal mechanical thresholds in naïve animals or on responses measured on the non-lesioned paw of CCI rats. Finally, TRO19622 had no effect on the response to noxious heat stimulus (52°C hot plate test) in either diabetic or normal rats (data not shown). Therefore, we believe that the anti-nociceptive effects of TRO19622 have different mechanism of action compared to current anti-convulsant, antidepressant, anesthetic or analgesic treatments and that this mechanism may distinguish syndromes like diabetic or chemotherapy-induced neuropathic pain from other types of chronic or acute pain.

Mitochondria as a therapeutic target in neuropathic pain

TRO19622 has been shown to bind to two outer mitochondrial membrane proteins, the peripheral benzodiazepine receptor (also called translocator protein 18 kDa) and the voltage-dependant anion channel (Bordet et al., 2007). These proteins are part of the mitochondrial permeability transition pore (mPTP) and may have relevance for pain control. Indeed recent findings put forward the hypothesis that mitochondrial dysfunction is present in both diabetic and chemotherapy-induced painful neuropathies (Lowell and Shulman, 2005; Flatters and Bennett, 2006; Leinninger et al., 2006; Siau and Bennett, 2006). Increased production of mitochondrial ROS in hyperglycemic cells is recognized as a major cause of the clinical complications associated with diabetes and is associated with mitochondrial fragmentation and apoptosis in neurons (Leinninger et al., 2006). In a rat model of chemotherapy-induced painful peripheral neuropathy using low doses of paclitaxel, the earliest sign of nerve dysfunction was the appearance of swollen and vacuolated mitochondria in both C-fibres and myelinated axons while there was no evidence of nerve degeneration nor changes in microtubule densities (Flatters and Bennett, 2006). In addition, these mitochondrial changes resolved when pain behavior decreased suggesting that abnormality in axonal mitochondria directly contributes to chemotherapy-induced pain. Similarly, peripheral nerve biopsies from patients suffering from vincristine-evoked painful neuropathy revealed axonal and mitochondrial swelling while microtubule alterations were not evidenced (Thant et al., 1982). Recently mitochondrial abnormalities have been linked to dysregulation of intracellular calcium homeostasis in the context of neuropathic pain. Intrathecal administration of calcium chelators significantly inhibited mechano-allodynia and mechano-hyperalgesia in rats treated with anti-human immunodeficiency virus nucleoside analog 2',3'-dideoxycytidine, paclitaxel or vincristine, whereas neuropathic pain caused by CCI was not reversed by calcium chelators ruling out dysregulated mitochondrial function as an underlying factor in this model (Joseph

et al., 2004; Siau and Bennett, 2006). Strikingly, TRO19622 was ineffective in both the formalin CCI and pain models, each partially involving inflammatory mediators (Kleinschnitz et al., 2004; Moalem and Tracey, 2006). Lack of effect in these models is consistent with previous findings (Bordet et al., 2007) that TRO19622 has no activity on nuclear steroid receptors such as estrogen, progesterone or glucocorticoid receptors that may modulate inflammation. Furthermore, TRO19622 did not prevent the phase of inflammation-driven neurodegeneration (first week post injury; Kleinschnitz et al., 2004) following a sciatic nerve crush (Bordet et al., 2007).

Microtubule-targeting drugs have been shown to cause mPTP opening (Evtodienko et al., 1996) through their interaction with the cytoskeleton. Indeed close association of beta-tubulin and the mPTP protein complex have been shown (Carre et al., 2002). Acetyl-L-carnitine (ALCAR) facilitates beta-oxidation, increases ATP production, prevents mitochondrial oxidative damage and mPTP opening and protects against various neurotoxic insults (Pastorino et al., 1993; Shigenaga et al., 1994). Treatment with ALCAR can both prevent and reverse the neuropathic pain syndrome evoked by paclitaxel (Pisano et al., 2003; Flatters et al., 2006). These effects were associated with the prevention of mitochondrial swelling in C-fibers while ALCAR had no neuroprotective effect on the degeneration of the intraepidermal terminal arbors of sensory neurons (Jin et al., 2008). By analogy, we hypothesize that TRO19622 preserves mitochondrial function in peripheral nerve through the control of the opening of the mPTP by interacting with VDAC and PBR in the same way as TRO19622 was found to prevent mitochondrial cytochrome c release in neurons undergoing apoptosis *in vitro* (Bordet et al., 2007). Acutely, these actions might rapidly correct metabolic imbalance in nerves to reverse hyperexcitability underlying allodynia and hyperalgesia associated with painful neuropathies while long term treatment may prevent or reverse nerve conduction deficits or axon degeneration. Future studies will explore these possibilities.

In conclusion, the results reported here along with previously described activity in other models of neurodegeneration suggest that TRO19622 may provide a novel approach to the treatment of neuropathic pain. We hypothesize that TRO19622 preserves mitochondrial function from stress induced by hyperglycemia or vincristine treatment, which may be especially important at nerve terminals that have high demands for ATP and calcium buffering. In addition, TRO19622 appears to have a good safety profile, lacking sedative or anesthetic effects that limit the use of some current drugs. Finally, the compound may provide a substantial benefit to patients over existing therapies by preventing or halting disease progression as well as relieving pain.

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REFERENCES

Ahlgren SC, Wang JF and Levine JD (1997) C-fiber mechanical stimulus-response functions are different in inflammatory versus neuropathic hyperalgesia in the rat. *Neuroscience* 76:285-290.

Amir R, Argoff CE, Bennett GJ, Cummins TR, Durieux ME, Gerner P, Gold MS, Porreca F and Strichartz GR (2006) The role of sodium channels in chronic inflammatory and neuropathic pain. *J Pain* 7:S1-29.

Andriambeloson E, Baillet C, Vitte PA, Garotta G, Dreano M and Callizot N (2006) Interleukin-6 attenuates the development of experimental diabetes-related neuropathy. *Neuropathology* 26:32-42.

Bennett GJ and Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107.

Bordet T, Buisson B, Michaud M, Drouot C, Galea P, Delaage P, Akentieva NP, Evers AS, Covey DF, Ostuni MA, Lacapere JJ, Massaad C, Schumacher M, Steidl EM, Maux D, Delaage M, Henderson CE and Pruss RM (2007) Identification and characterization of cholest-4-en-3-one, oxime (TRO19622), a novel drug candidate for amyotrophic lateral sclerosis. *J Pharmacol Exp Ther* 322:709-720.

Calcutt NA, Jorge MC, Yaksh TL and Chaplan SR (1996) Tactile allodynia and formalin hyperalgesia in streptozotocin-diabetic rats: effects of insulin, aldose reductase inhibition and lidocaine. *Pain* 68:293-299.

Carre M, Andre N, Carles G, Borghi H, Brichese L, Briand C and Braguer D (2002) Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel. *J Biol Chem* 277:33664-33669.

Cata JP, Weng HR, Lee BN, Reuben JM and Dougherty PM (2006) Clinical and experimental findings in humans and animals with chemotherapy-induced peripheral neuropathy. *Minerva Anestesiol* 72:151-169.

Chen X and Levine JD (2007) Mechanically-evoked C-fiber activity in painful alcohol and AIDS therapy neuropathy in the rat. *Mol Pain* 3:5.

Courteix C, Eschalier A and Lavarenne J (1993) Streptozocin-induced diabetic rats: behavioural evidence for a model of chronic pain. *Pain* 53:81-88.

Cundy KC, Annamalai T, Bu L, De Vera J, Estrela J, Luo W, Shirsat P, Torneros A, Yao F, Zou J, Barrett RW and Gallop MA (2004) XP13512 [(+/-)-1-([(alpha-isobutanoyloxyethoxy)carbonyl] aminomethyl)-1-cyclohexane acetic acid], a novel gabapentin prodrug: II. Improved oral bioavailability, dose proportionality, and colonic absorption compared with gabapentin in rats and monkeys. *J Pharmacol Exp Ther* 311:324-333.

Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice AS, Stacey BR, Treede RD, Turk DC and Wallace MS (2007) Pharmacologic management of neuropathic pain: evidence-based recommendations. *Pain* 132:237-251.

Evtodienko YV, Teplova VV, Sidash SS, Ichas F and Mazat JP (1996) Microtubule-active drugs suppress the closure of the permeability transition pore in tumour mitochondria. *FEBS Lett* 393:86-88.

Fernyhough P, Diemel LT, Hardy J, Brewster WJ, Mohiuddin L and Tomlinson DR (1995) Human recombinant nerve growth factor replaces deficient neurotrophic support in the diabetic rat. *Eur J Neurosci* 7:1107-1110.

Fernyhough P, Diemel LT and Tomlinson DR (1998) Target tissue production and axonal transport of neurotrophin-3 are reduced in streptozotocin-diabetic rats. *Diabetologia* 41:300-306.

Flatters SJ and Bennett GJ (2006) Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: evidence for mitochondrial dysfunction. *Pain* 122:245-257.

Flatters SJ, Xiao WH and Bennett GJ (2006) Acetyl-L-carnitine prevents and reduces paclitaxel-induced painful peripheral neuropathy. *Neurosci Lett* 397:219-223.

Hanaoka Y, Ohi T, Furukawa S, Furukawa Y, Hayashi K and Matsukura S (1992) Effect of 4-methylcatechol on sciatic nerve growth factor level and motor nerve conduction velocity in experimental diabetic neuropathic process in rats. *Exp Neurol* 115:292-296.

Hanaoka Y, Ohi T, Furukawa S, Furukawa Y, Hayashi K and Matsukura S (1994) The therapeutic effects of 4-methylcatechol, a stimulator of endogenous nerve growth factor synthesis, on experimental diabetic neuropathy in rats. *J Neurol Sci* 122:28-32.

Hellweg R, Raivich G, Hartung HD, Hock C and Kreutzberg GW (1994) Axonal transport of endogenous nerve growth factor (NGF) and NGF receptor in experimental diabetic neuropathy. *Exp Neurol* 130:24-30.

Hong S, Morrow TJ, Paulson PE, Isom LL and Wiley JW (2004) Early painful diabetic neuropathy is associated with differential changes in tetrodotoxin-sensitive and -resistant sodium channels in dorsal root ganglion neurons in the rat. *J Biol Chem* 279:29341-29350.

Jin HW, Flatters SJ, Xiao WH, Mulhern HL and Bennett GJ (2008) Prevention of paclitaxel-evoked painful peripheral neuropathy by acetyl-L-carnitine: effects on axonal mitochondria, sensory nerve fiber terminal arbors, and cutaneous Langerhans cells. *Exp Neurol* 210:229-237.

Joseph EK, Chen X, Khasar SG and Levine JD (2004) Novel mechanism of enhanced nociception in a model of AIDS therapy-induced painful peripheral neuropathy in the rat. *Pain* 107:147-158.

Kleinschnitz C, Brinkhoff J, Zelenka M, Sommer C and Stoll G (2004) The extent of cytokine induction in peripheral nerve lesions depends on the mode of injury and NMDA receptor signaling. *J Neuroimmunol* 149:77-83.

Leinninger GM, Edwards JL, Lipshaw MJ and Feldman EL (2006) Mechanisms of disease: mitochondria as new therapeutic targets in diabetic neuropathy. *Nat Clin Pract Neurol* 2:620-628.

Lowell BB and Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384-387.

Luo ZD, Calcutt NA, Higuera ES, Valder CR, Song YH, Svensson CI and Myers RR (2002) Injury type-specific calcium channel alpha 2 delta-1 subunit up-regulation in rat neuropathic pain models correlates with antiallodynic effects of gabapentin. *J Pharmacol Exp Ther* 303:1199-1205.

Malcangio M and Tomlinson DR (1998) A pharmacologic analysis of mechanical hyperalgesia in streptozotocin/diabetic rats. *Pain* 76:151-157.

Mizisin AP, Calcutt NA, Tomlinson DR, Gallagher A and Fernyhough P (1999) Neurotrophin-3 reverses nerve conduction velocity deficits in streptozotocin-diabetic rats. *J Peripher Nerv Syst* 4:211-221.

Moalem G and Tracey DJ (2006) Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev* 51:240-264.

Pastorino JG, Snyder JW, Serroni A, Hoek JB and Farber JL (1993) Cyclosporin and carnitine prevent the anoxic death of cultured hepatocytes by inhibiting the mitochondrial permeability transition. *J Biol Chem* 268:13791-13798.

Pisano C, Pratesi G, Laccabue D, Zunino F, Lo Giudice P, Bellucci A, Pacifici L, Camerini B, Vesci L, Castorina M, Cicuzza S, Tredici G, Marmiroli P, Nicolini G, Galbiati S, Calvani M, Carminati P and Cavaletti G (2003) Paclitaxel and Cisplatin-induced neurotoxicity: a protective role of acetyl-L-carnitine. *Clin Cancer Res* 9:5756-5767.

Said G (2007) Diabetic neuropathy--a review. *Nat Clin Pract Neurol* 3:331-340.

Shigenaga MK, Hagen TM and Ames BN (1994) Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci U S A* 91:10771-10778.

Siau C and Bennett GJ (2006) Dysregulation of cellular calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy. *Anesth Analg* 102:1485-1490.

Siau C, Xiao W and Bennett GJ (2006) Paclitaxel- and vincristine-evoked painful peripheral neuropathies: loss of epidermal innervation and activation of Langerhans cells. *Exp Neurol* 201:507-514.

Thant M, Hawley RJ, Smith MT, Cohen MH, Minna JD, Bunn PA, Ihde DC, West W and Matthews MJ (1982) Possible enhancement of vincristine neuropathy by VP-16. *Cancer* 49:859-864.

Tomlinson DR and Gardiner NJ (2008) Glucose neurotoxicity. *Nat Rev Neurosci* 9:36-45.

Vincent AM, Russell JW, Low P and Feldman EL (2004) Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr Rev* 25:612-628.

Visovsky C (2003) Chemotherapy-induced peripheral neuropathy. *Cancer Invest* 21:439-451.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16:109-110.

FOOTNOTES

Correspondence and reprint requests to:

Dr. Rebecca M. Pruss, Trophos, Parc Scientifique de Luminy, Luminy Biotech Entreprises, Case 931, 13288 Marseille Cedex 9, France. Tel +33 491 828 281; Fax +33 491 828 289; E-mail: rpruss@trophos.com

Present address (B.B.) NEUROSERVICE, bât. INMED, 163 avenue de Luminy, 13273 Marseille cedex 09, FRANCE

LEGENDS FOR FIGURES

Figure 1. Effects of TRO19622 administration on (A) latency of the CMAP and (B) sensory nerve conduction in diabetic rats. Diabetes was induced in Sprague-Dawley rats by a single intravenous (i.v.) injection of streptozotocin (STZ) on day 0. Animals were next treated daily from day 10 to day 40 with vehicle (corn oil, 5 ml/kg), TRO19622 (3, 30 and 300 mg/kg, p.o.) or 4-methylcathecol (4-MC) (10 µg/ml, i.p.). (A-B) Electrophysiological recordings were performed on days 8 and 40 post-STZ to assess effects of diabetes on nerve conduction. (A) Latency of the compound muscle action potential (CMAP) and (B) the sensory nerve conduction velocity (SNCV) were recorded using a Neuromatic 2000M electromyograph on anesthetized rats maintained with a heating lamp and controlled by a contact thermometer. Values are means ± s.e.m. (n = 12/group). *$p < 0.05$, ***$p < 0.001$ as compared to the STZ/vehicle group.

Figure 2. Acute (A) and repeated (B) oral administration of TRO19622 reversed mechanical allodynia in diabetic rats. Mechanical allodynia was assessed in Wistar rats by monitoring the paw withdrawal threshold using a Dynamic Plantar Aesthesiometer. Baseline (BL) measurements were performed before STZ injection and five weeks after STZ injection when significant allodynia was established. Animals were then treated for 5 consecutive days with oral administration of vehicle (corn oil, 5 ml/kg), TRO19622 (10, 30 or 100 mg/kg once a day) or with gabapentin (GBP; 50 mg/kg twice a day). Paw withdrawal thresholds were measured 4 hours after TRO19622 or vehicle and 3 hours after the morning dose of gabapentin. Values are means ± s.e.m. (n = 7-8/group). † $p < 0.005$, 5 wks post STZ values versus baseline values; *$p < 0.05$ as compared to the STZ-vehicle group at same day.

Figure 3. Acute (A) and repeated (B) oral administration of TRO19622 reversed mechanical allodynia in rats suffering from vincristine-induced neuropathy. Mechanical allodynia was assessed in Wistar rats by monitoring the paw withdrawal threshold using a Dynamic Plantar Aesthesiometer. Baseline measurements were performed on day 0 before animals received i.v. injections of vincristine on days 1, 4 and 6. Testing was performed on days 3, 5 and 7 when allodynia was fully established. Starting on day 10, animals were treated for 5 consecutive days by oral administration of vehicle (corn oil, 5 ml/kg), TRO19622 (10, 30 or 100 mg/kg) or gabapentin (50 mg/kg, twice a day). Testing was repeated on days 10 to 14, 4 hours after TRO19622 or vehicle and 3 hours after the morning dose of gabapentin. Values are means ± s.e.m. (n = 8/group). †p <0.001 versus day 0 values; *p < 0.05, **p < 0.01, ***p < 0.001 as compared to the vehicle group at same day.

Figure 4. TRO19622 administration did not reverse mechanical allodynia or alter mechanical thresholds in rats following a chronic constriction injury to the sciatic nerve. Mechanical allodynia following CCI in the lesioned hind paw (A) or mechanical threshold in the sham operated hind paw (B) were assessed in Wistar rats using an Electronic Von Frey Aesthesiometer. Baseline measurements were taken before surgery (BL) and before treatment (day 0). Animals were then treated daily for 5 consecutive days with TRO19622 (3, 30 or 300 mg/kg, p.o.), vehicle (corn oil, 5 ml/kg) or gabapentin (200 mg/kg, p.o.) and paw withdrawal thresholds assessed each day 4 hours after TRO19622 and vehicle treatment or 1 hr after gabapentin dosing. Values are means ± s.e.m. (n = 6/group).). † p < 0.001 versus BL values; ***p < 0.001 as compared to the vehicle group.

Figure 5. Acute administration of TRO19622 did not reversed mechanical allodynia and hyperalgesia in naïve rats. Behavioral thresholds to mechanical stimulation were assessed in

Wistar rats before treatment (white columns) and 4 hours after (black columns) oral administration of vehicle (corn oil, 5mL/kg) or TRO19622 (100 mg/kg) using the Dynamic Plantar Aesthesiometer and the Analgesimeter (Ugo Basile, Italy) as described in Methods. Values are means ± s.e.m. (n = 7-8/group).

Table 1. Body weight and blood glucose levels in control and STZ-diabetic rats

Values are means ± s.e.m. (n = 12-13 rats).

Study groups	Daily doses (from D10 to D40)	Body weight (g)			Blood glucose concentration (mg/dL)	
		D0	D 8	D 40	D 8	D 40
Control/Vehicle	5 mL/kg	248 ± 6	328 ± 9	456 ± 26	117 ± 3	100 ± 2
STZ/Vehicle	5 mL/kg	247 ± 8	263 ± 18	289 ± 50	544 ± 24	574 ± 16
STZ/TRO19622	3 mg/kg	253 ± 5	247 ± 24	273 ± 54	543 ± 17	553 ± 36
	30 mg/kg	255 ± 6	264 ± 15	301 ± 43	517 ± 19	524 ± 20
	300 mg/kg	252 ± 9	269 ± 27	296 ± 41	526 ± 16	582 ± 14
STZ/4-MC	10 µg/kg	253 ± 5	255 ± 16	281 ± 39	526 ± 18	580 ± 10

D, day post- STZ injection

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Table 2. Pain behavior in STZ-diabetic rats

Sprague-Dawley rats used in the nerve conduction study were used to assess pain behavior on days 10 and 20 post-STZ. Thermal allodynia on day 10 was assessed in the 38°C hot plate test 4h after the first administration of TRO19622 or vehicle (corn oil) or 45 min following subcutaneous administration of morphine as described in Methods. A cut-off time was set to 30 s. On day 20, the effect of 10 days repeated treatment with TRO19622, vehicle or 4-MC on mechanical hyperalgesia was assessed using the Randall-Sellito paw pressure test. Animals treated with 4-MC were also tested on day 21 to assess the anti-nociceptive effect of acute administration of morphine. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to the STZ/vehicle group.

Study groups	Thermal allodynia, D10		Mechanical hyperalgesia, D20 or D21
	Animals reaching cut-off	Response time Mean ± sem (s)	Paw withdrawal threshold Mean ± sem (g)
Control/Vehicle	10/12	29.2 ± 0.7**	175.8 ± 10***
STZ/Vehicle	4/13	19.4 ± 2.5	79.2 ± 5.5
STZ/3 mg/kg TRO19622	2/13	19.2 ± 2.1	88.5 ± 6.4
STZ/30 mg/kg TRO19622	7/13	25.2 ± 2.1[a]	113.5 ± 7.7*
STZ/300 mg/kg TRO19622	8/13	26.2 ± 1.8[a]	110.4 ± 9.1*
STZ/10 µg/kg 4-MC	ND	ND	80.4 ± 8.9
STZ/4-MC/3 mg/kg morphine	9/13	27.6 ± 1.3**	131.2 ± 6.8***

D, day post-STZ injection; ND, not determined

[a] $p < 0.05$ using Fisher's Least Significant Difference Test

Table 3. Responses in the formalin test

Test substances were administered orally to groups of 6 adult Wistar rats 60 minutes (morphine) or 4 hours (TRO19622 or vehicle) before intraplantar injection of formalin (0.05 ml, 5% solution). The paw licking time was recorded in 5 minute intervals; 0 to 5 minutes corresponded to the first phase and 15 to 30 minutes corresponded to the second phase. Data shown are mean ± s.e.m. of the licking time (seconds) and as percent reduction compared to vehicle-treated group. ** $p < 0.01$, *** $p < 0.001$ versus vehicle-treated animals.

Treatment	First Phase (1-5 min)		Second Phase (15-30 min)	
	Time (s)	% reduction	Time (s)	% reduction
Vehicle	60.5 ± 5.5	-	125 ± 23.6	-
TRO19622 300 mg/kg, p.o.	51.5 ± 7.5	15 ± 12	174.5 ± 27.9	-40 ± 22
Morphine 60 mg/kg, p.o.	15.2 ± 5.1***	75 ± 8	5.5 ± 5.1**	96 ± 4

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Table 4. TRO19622 plasma concentrations in rats at the end of various pain behavior and pharmacokinetic studies

Plasma TRO19622 concentrations were determined at the end of each study (following the final testing session) approximately 4h after the last administration of TRO19622. Values are means ± s.e.m. (n = 3-6 rats).

Study	TRO19622 treatment		TRO19622 plasma concentration	
	Treatment duration	Dose mg/kg	µM	µg/mL
STZ (nerve conduction study)	32d	3	0.8 ± 0.3	0.3 ± 0.1
		30	8.4 ± 3.3	3.3 ± 1.3
		300	> 50	> 20
STZ (pain study)	5d	10	4.5 ± 1.8	1.8 ± 0.7
		30	8.1 ± 2.4	3.3 ± 0.9
		100	37.5 ± 7.3	15.0 ± 2.9
Vincristine	5d	10	2 ± 1.3	0.8 ± 0.5
		30	3.9 ± 1.1	1.6 ± 0.4
		100	14.2 ± 1.4	5.7 ± 0.6
CCI	5d	3	0.5 ± 0.2	0.2 ± 0.1
		30	4.2 ± 1.5	1.7 ± 0.6
		300	24.3 ± 0.5	9.7 ± 2.0
Formalin	acute	300	5.5 ± 2.1	2.2 ± 0.8
Pharmacokinetics	acute	10	0.7 ± 0.2	0.3 ± 0.1
		100	8.6 ± 1.2	3.4 ± 0.5

Figure 1

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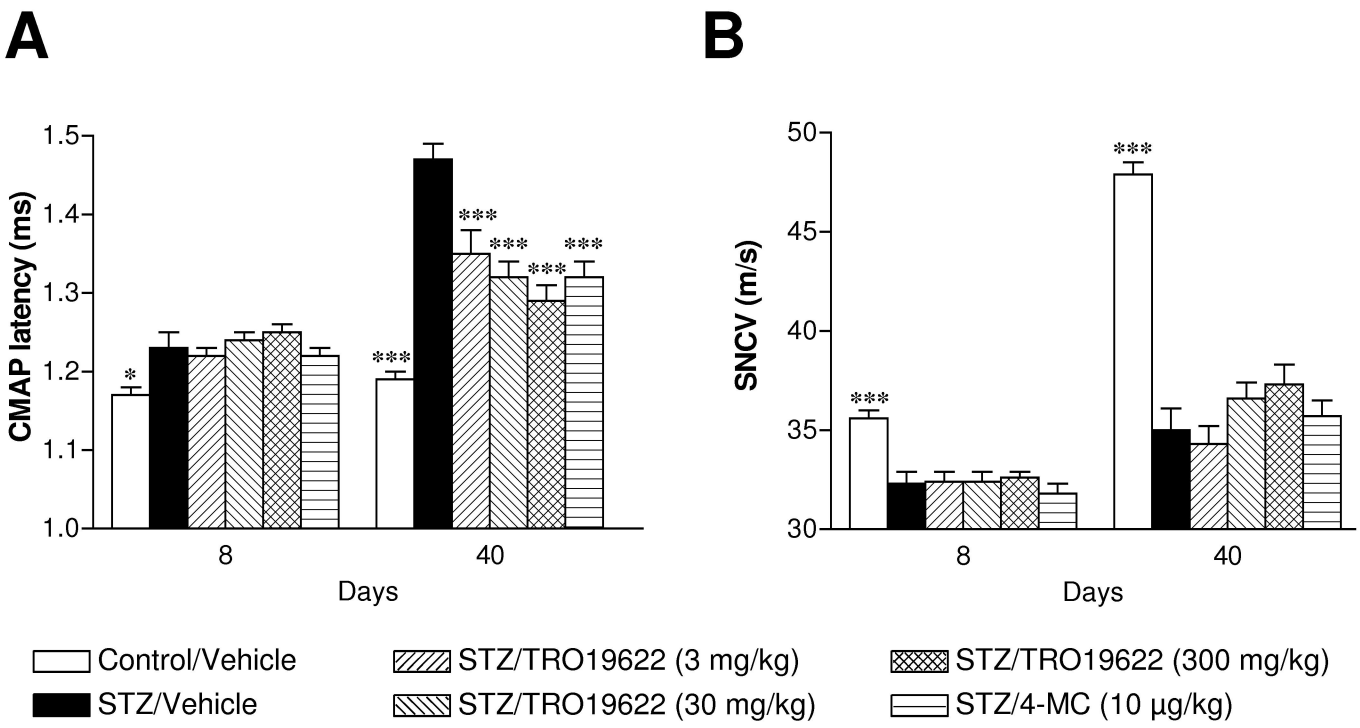

Figure 2

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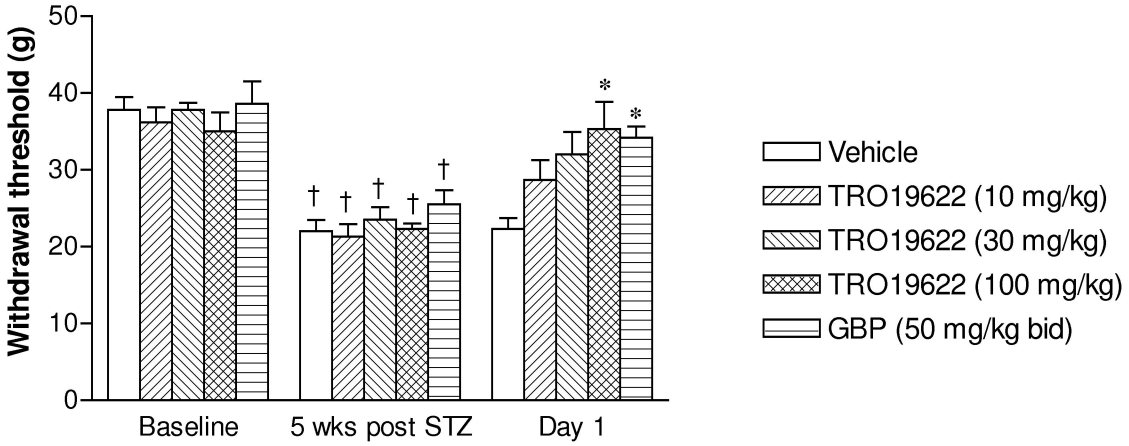

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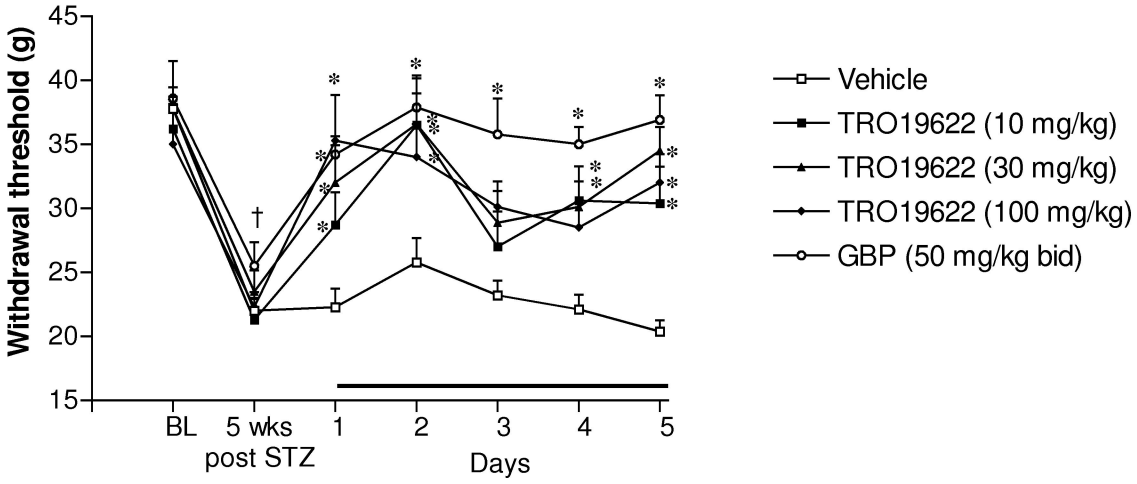

Figure 3

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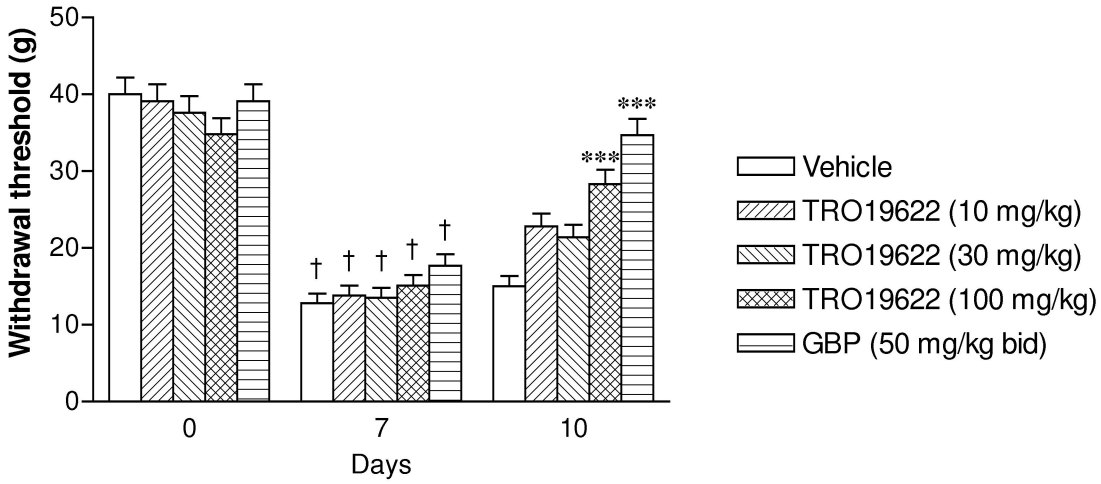

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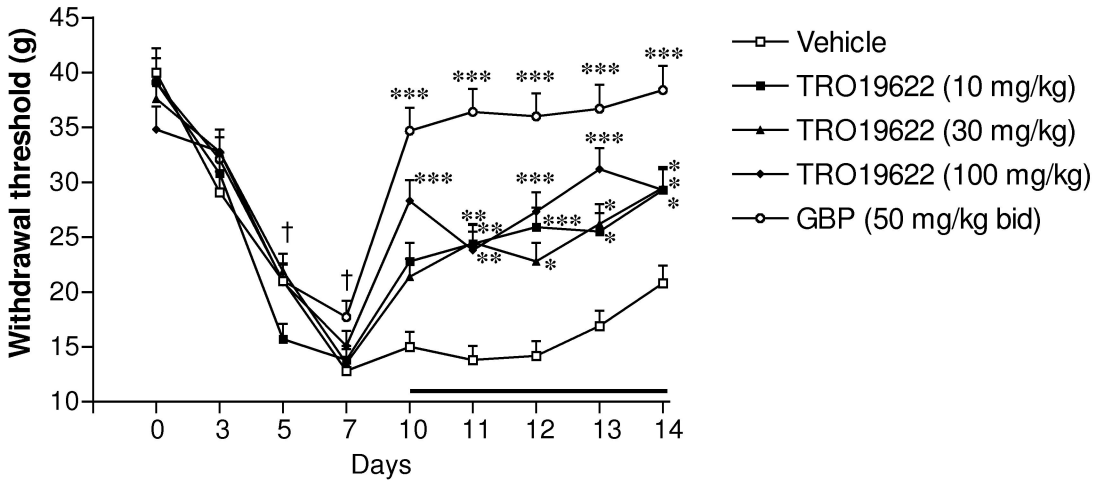

Figure 4

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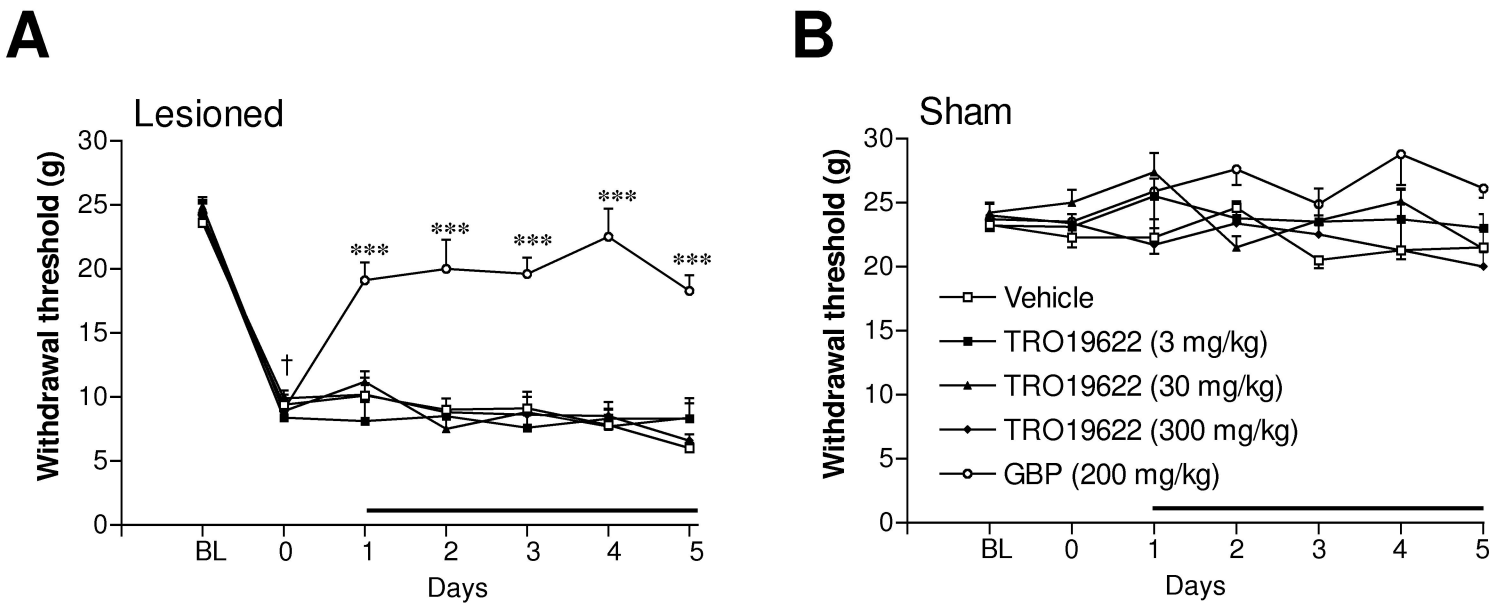

Figure 5

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