

Specific Antinociceptive Activity of Cholest-4-en-3-one, Oxime (TRO19622) in Experimental Models of Painful Diabetic and Chemotherapy-Induced Neuropathy^S

Thierry Bordet, Bruno Buisson,¹ Magali Michaud, Jean-Louis Abitbol, Fabien Marchand, John Grist, Emile Andriambelison, Marzia Malcangio, and Rebecca M. Pruss

Trophos, Parc Scientifique de Luminy, Luminy Biotech Entreprise, Case 931, Marseille, France (T.B., B.B., M.Mi., J.-L.A., R.M.P.); Neurorestoration Group Wolfson CARD, King's College London, London, United Kingdom (F.M., J.G., M.Ma.); and Neurofit, Parc d'Innovation, Illkirch, France (E.A.)

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, that 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 neuro-

pathic pain behavior as early as the first administration. Further exploration of these acute antinociceptive effects demonstrated that TRO19622 was also able to reverse tactile allodynia in vincristine-treated rats, a model of chemotherapy-induced neuropathic pain. It is interesting to note that 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-driven, or lesion-induced neuropathic pain. These results support the potential use of TRO19622 to treat painful diabetic and chemotherapy-induced neuropathies.

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 postherpetic 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, anticonvulsants, 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 antiretroviral 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, 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 (for reviews, see Vincent et al.,

¹ Current affiliation: NEUROSERVICE, Marseille, France.

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ABBREVIATIONS: TRO19622, cholest-4-en-3-one, oxime; STZ, streptozotocin; CCI, chronic constriction injury; 4-MC, 4-methylcatechol; SNCV, sensory nerve conduction velocity; CMAP, compound muscle action potential; NGF, nerve growth factor; mPTP, mitochondrial permeability transition pore; ALCAR, acetyl-L-carnitine.

2004; Leininger et al., 2006; Tomlinson and Gardiner, 2008). Chemotherapy with platins, taxanes, or vinca alkaloids induces neuropathic pain behavior in rats that mimics that in seen in patients (Cata et al., 2006). Although vincristine and paclitaxel inhibit tumor cell proliferation by disrupting microtubule polymerization, neuropathic pain behavior occurs 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, 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 antinociceptive effects of TRO19622 shown in this article 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 Limited (Bicester, Oxon, UK) or from BioLasco Taiwan (Taipei, Taiwan). Rats were housed under temperature-controlled (21–24°C) and 12-h 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 Association for the Study of Pain (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 i.v. or i.p. injection of STZ (55 mg/kg; Sigma Chemical, Poole, Dorset, UK). Animals showing blood glucose levels above 260 mg/dl (15 mmol/l) approximately 1 week after STZ injection were considered hyperglycemic and diabetic.

Induction of Neuropathic Pain with Vincristine. Vincristine sulfate (Sigma Chemical) was dissolved in 0.9% NaCl, and Wistar rats (~250 g) received injections with 200 µg/kg i.v. (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 Chemical) was dissolved in 0.9% NaCl and vortexed until complete dissolution. Intraperitoneal administration with 10 µg/kg 4-MC was performed daily with a dosage volume of 1 ml/kg.

Morphine (except as noted below) was dissolved in 0.9% NaCl, vortexed until complete dissolution, and administered subcutane-

ously 45 min before 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, CA), 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 before (day -3) and on days 8, 25, and 40 post-STZ injection using electrophysiological measurements, as described previously in this model (Andriambeloso et al., 2006). Rats were anesthetized by i.p. injection of 60 mg/kg ketamine hydrochloride (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, toward 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 meters per second. 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 milliseconds. 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 to avoid any uncontrollable environmental influence that might introduce variability in behavioral responses. Baseline responses were recorded in each animal before surgery, STZ injection, or vincristine injection.

Assessment of Thermal Allodynia and Mechanical Hyperalgesia in Rats Receiving STZ Injections. Thermal allodynia was assessed using a warm (38°C) plate as described previously in this model (Andriambeloso et al., 2006). In brief, 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 cut-off time of 30 s.

Mechanical hyperalgesia was assessed with the Randall and Sellito test using a paw pressure analgesimeter (Bioseb, Vitrolles, 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 4 h after the administration of TRO19622, 4-MC, or vehicle (corn oil), or 45 min after injection of morphine.

Assessment of Tactile Allodynia in Rats Receiving STZ and Vincristine Injections. Tactile allodynia was measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy). In brief, each animal was placed in a clear acrylic cubicle (22 × 16.5 × 14 cm) with a metal grid floor giving access to the underside of their paws, and they were 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 (0.5 mm in diameter) exerting a linearly increasing force (2.5 g/s). The force (grams) 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 50 g was imposed to prevent tissue damage. Animals received injections with STZ were tested once a week from week 3 to week 5, and those displaying significant allodynia on week 5 compared with 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 4 h after administration of TRO19622 or vehicle and 3 h 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 in 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) approximately 1 mm 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 min. Responsiveness to a #12 Supertip (IITC Life Science, Woodland Hills, CA) applied beneath the mesh floor perpendicular to the central plantar surface of the hind paw was assessed on both the lesioned and nonlesioned 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; IITC Life Science). 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 before nerve ligation. Test compounds were administered once a day for five consecutive days by oral gavage to groups of five animals and testing for tactile allodynia was performed 1 h after gabapentin (200 mg/kg in 2% Tween 80; 10 ml/kg; Pfizer Central Research, Sandwich, Kent, UK) or 4 h after vehicle (corn oil) or TRO19622 (3, 30, or 300 mg/kg). Although 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 before use. TRO19622 (300 mg/kg) and morphine (60 mg/kg; 10 ml/kg) were administered by oral gavage to groups of six animals 4 or 1 h, respectively, before subplantar injection of formalin (0.05 ml; 5% solution) in one hind paw. Hind paw licking time (seconds) was measured at 5-min intervals for 30 min after formalin injection. Values from 0 to 5 min and from 15 to 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 three to six animals per group ~4 h after the last administration. The blood was collected in lithium-heparin tubes, then it was centrifuged at 1200 rpm to obtain

plasma, which was stored frozen until analysis by high-performance liquid chromatography-tandem mass spectrometry.

Statistical Analysis. Results were expressed as mean \pm S.E.M. Statistical analysis was assessed by analysis of variance 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 1 week following a single injection of STZ, blood glucose levels in diabetic rats were 4 to 5 times higher compared with saline-treated rats (544 ± 24 mg/dl in STZ rats versus 117 ± 3 mg/dl in controls), and they 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 rats receiving STZ injections, whereas it increased steadily in control rats (from 248 ± 6 to 456 ± 26 g in controls versus from 247 ± 8 to 289 ± 50 g in STZ rats, respectively; days 0 and 40). Daily treatment with TRO19622 or 4-MC had no effect on hyperglycemia or body weight of rats that received STZ injections (Table 1).

Similar to the effects seen after 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 to 65% was seen over the dose range tested (3–300 mg/kg/day) compared with vehicle-treated rats, and treatment with the positive control, 4-MC, had a similar effect on CMAP latency as the 30 mg/kg/day dose of TRO19622 (Fig. 1A). Despite these effects on motor nerve conduction, neither 4-MC nor TRO19622 treatment significantly altered SNCV dysfunction in diabetic rats by day 40 (Fig. 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 after STZ injection was assessed 4 h after a single oral administration. Second, the effects of prolonged treatment (10 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 before 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

TABLE 1

Body weight and blood glucose levels in control and STZ-diabetic rats
Values are means \pm S.E.M. (*n* = 12–13 rats).

Study Group	Daily Dose (from D10–D40)	Body Wt			Blood Glucose Conc.	
		D0	D8	D40	D 8	D 40
		<i>g</i>			<i>mg/dl</i>	
Control/vehicle	5 ml/kg	248 \pm 6	328 \pm 9	456 \pm 26	117 \pm 3	100 \pm 2
STZ/vehicle	5 ml/kg	247 \pm 8	263 \pm 18	289 \pm 50	544 \pm 24	574 \pm 16
STZ/TRO19622	3 mg/kg	253 \pm 5	247 \pm 24	273 \pm 54	543 \pm 17	553 \pm 36
	30 mg/kg	255 \pm 6	264 \pm 15	301 \pm 43	517 \pm 19	524 \pm 20
	300 mg/kg	252 \pm 9	269 \pm 27	296 \pm 41	526 \pm 16	582 \pm 14
STZ/4-MC	10 μ g/kg	253 \pm 5	255 \pm 16	281 \pm 39	526 \pm 18	580 \pm 10

D, day post-STZ injection.

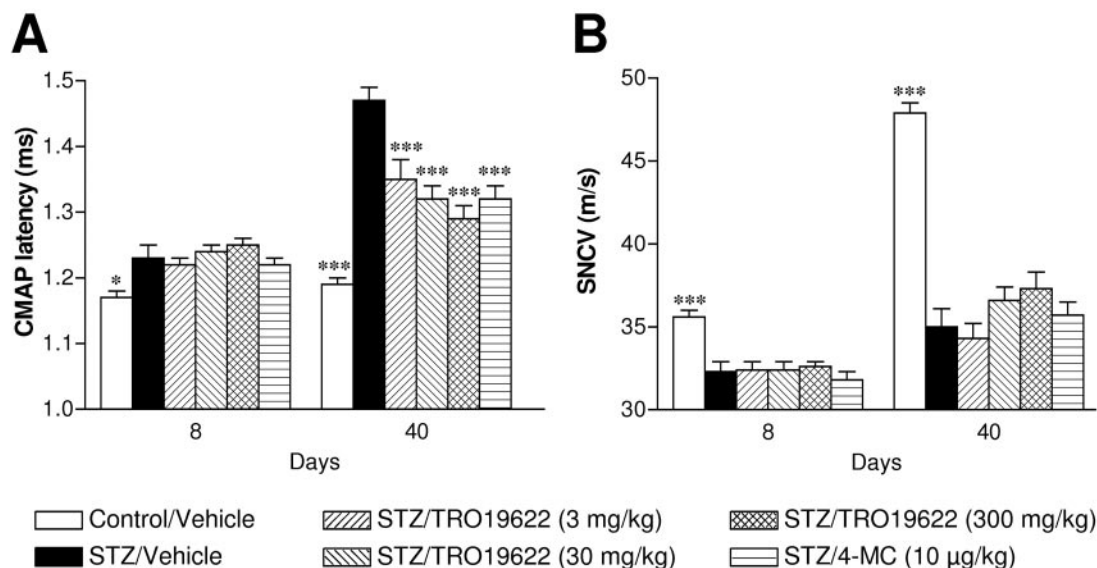


Fig. 1. Effects of TRO19622 administration on latency of the CMAP (A) and sensory nerve conduction (B) in diabetic rats. Diabetes was induced in Sprague-Dawley rats by a single i.v. injection of STZ on day 0. Animals were next treated daily from day 10 to day 40 with vehicle (corn oil, 5 ml/kg), TRO19622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

TRO19622 to reverse STZ thermal allodynia 4 h after 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 compared with control animals (79.2 ± 5.5 g in STZ-vehicle rats versus 175.8 ± 10 in controls; $p < 0.001$; Table 2). Although 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 with vehicle-treated diabetic rats (113.5 ± 7.7 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; $p > 0.05$). Because there was no difference in glycemia or body weight between groups of rats receiving

STZ injections in this study (see above), the antinociceptive 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 antiallodynic 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 described previously, STZ-induced diabetes produced a significant decrease in the threshold to mechanical stimulation that is fully developed 5 weeks after STZ injection ($p < 0.05$; Fig. 2). It is remarkable that 4 h 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; Fig. 2A). Repeated administration of TRO19622 for five consecutive days produced significant antiallodynic 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 with vehicle-

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 4 h after the first administration of TRO19622 or vehicle (corn oil) or 45 min following subcutaneous administration of morphine as described under *Materials and 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 antinociceptive effect of acute administration of morphine. Values are mean \pm S.E.M.

Study Group	Thermal Allodynia, D10		Mechanical Hyperalgesia, D20 or D21 Paw Withdrawal Threshold
	Animals Reaching Cut-Off	Response Time	
Control/vehicle	10/12	29.2 \pm 0.7**	175.8 \pm 10***
STZ/vehicle	4/13	19.4 \pm 2.5	79.2 \pm 5.5
STZ/3 mg/kg TRO19622	2/13	19.2 \pm 2.1	88.5 \pm 6.4
STZ/30 mg/kg TRO19622	7/13	25.2 \pm 2.1 ^a	113.5 \pm 7.7*
STZ/300 mg/kg TRO19622	8/13	26.2 \pm 1.8 ^a	110.4 \pm 9.1*
STZ/10 µg/kg 4-MC	N.D.	N.D.	80.4 \pm 8.9
STZ/4-MC/3 mg/kg morphine	9/13	27.6 \pm 1.3**	131.2 \pm 6.8***

D, day post-STZ injection; N.D., not determined.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the STZ/vehicle group.

^a $p < 0.05$ using Fisher's least significant difference test.

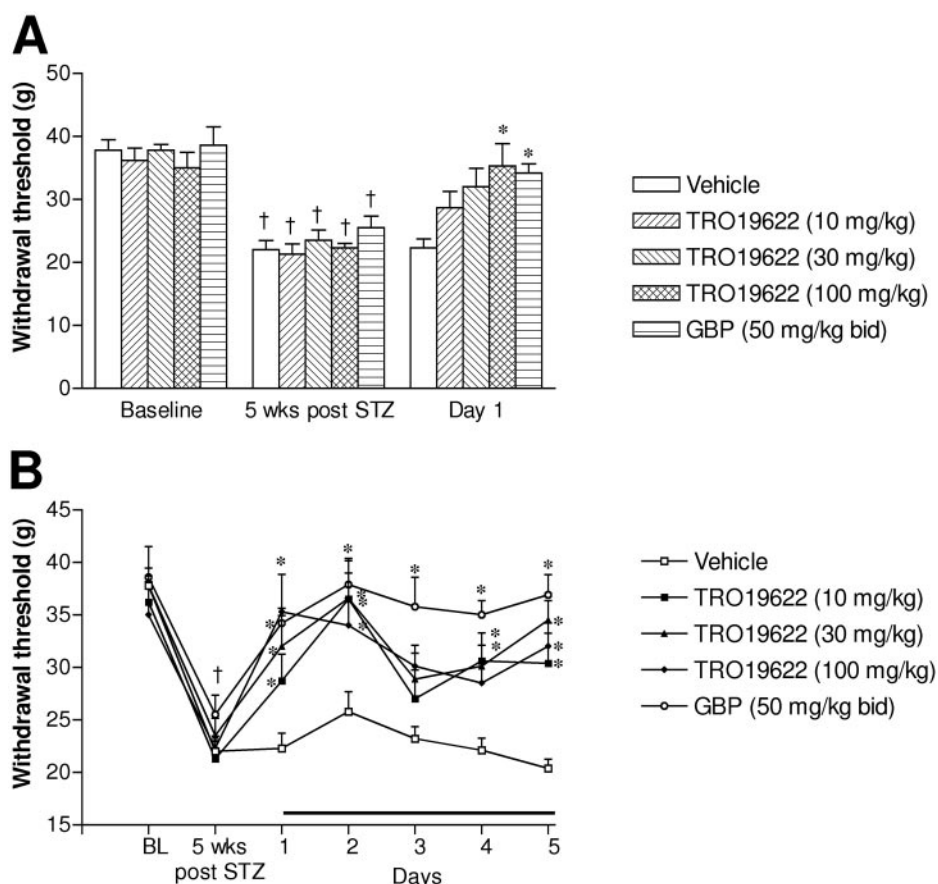


Fig. 2. Acute (A) and repeated (B) oral administration of TRO19622 reversed mechanical allodynia in diabetic rats. Mechanical allodynia was assessed in Wistar rats by monitoring the paw withdrawal threshold using a dynamic plantar aesthesiometer. Baseline (BL) measurements were performed before STZ injection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

treated rats ($p < 0.05$; Fig. 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 antinociceptive effect of TRO19622 was next evaluated in a rat model of vincristine-induced neuropathic pain. Rats developed tactile allodynia within 2 days after the first injection of vincristine (Fig. 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 (days 7–14; $p < 0.001$). As in diabetic rats, TRO19622 significantly reversed vincristine-induced allodynia 4 h after the first oral administration of the highest dose tested (100 mg/kg; day 10; $p < 0.001$; Fig. 3A). Although lower doses did not produce a significant antiallodynic 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 with vehicle-treated animals (Fig. 3B). Gabapentin (50 mg/kg twice a day) also effectively reversed tactile allodynia on days 10 to 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/day was studied on five consecutive days starting 7 days after 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, whereas gabapen-

tin (200 mg/kg/day) fully reversed allodynia on all days (Fig. 4A). In this study, potential sedative or anesthetic effects of TRO19622 were ruled out because there was no change in responses measured in the nonlesioned hind paw even at the highest dose of 300 mg/kg (Fig. 4B). Likewise, TRO19622 (100 mg/kg) had no effect on paw withdrawal thresholds of normal rats in tests of mechanical allodynia or mechanical hyperalgesia (Fig. 5). TRO19622 was also inactive in the formalin test; a single oral administration of 300 mg/kg TRO19622 4 h before subplantar injection of formalin had no significant effect on either the first or second phase of the pain response, whereas morphine (60 mg/kg) was efficacious in both phases of this model (Table 3).

Relationship between TRO19622 Plasma Concentration and Antinociceptive Efficacy. Plasma TRO19622 concentrations were determined at the end of each study (after the final testing session), approximately 4 h after the last administration of TRO19622 (Table 4). With the exception of 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 24 h, 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-day repeated administration of 300 mg/kg TRO19622 compared with that found 4 h after a single administration in the formalin test. It

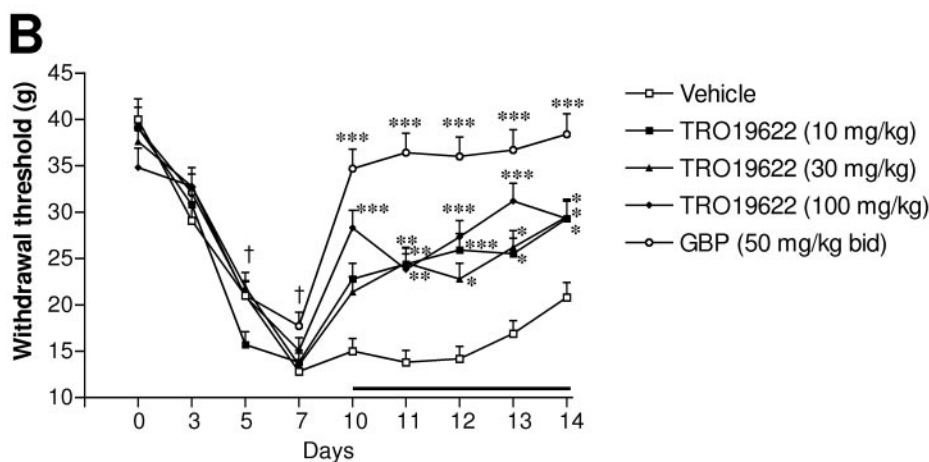
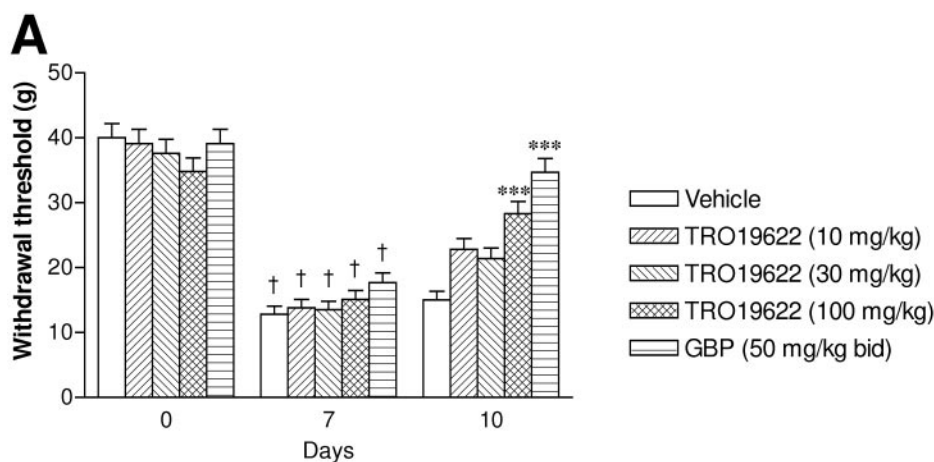


Fig. 3. Acute (A) and repeated (B) oral administration of TRO19622 reversed mechanical allodynia in rats suffering from vincristine-induced neuropathy. Mechanical allodynia was assessed in Wistar rats by monitoring the paw withdrawal threshold using a dynamic plantar aesthesiometer. Baseline measurements 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 five 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 from day 10 to day 14, 4 h after TRO19622 or vehicle and 3 h after the morning dose of gabapentin. Values are means \pm S.E.M. ($n = 8/\text{group}$). †, $p < 0.001$ versus day 0 values; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the vehicle group at same day.

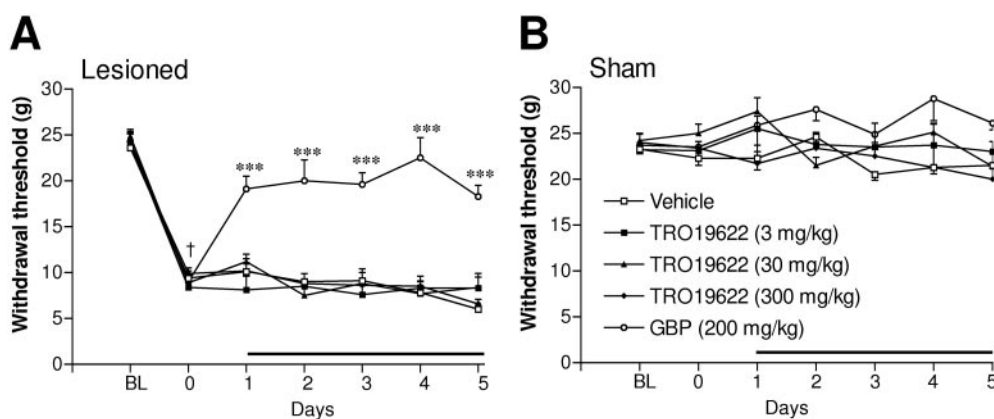


Fig. 4. TRO19622 administration did not reverse mechanical allodynia or alter mechanical thresholds in rats after a chronic constriction injury to the sciatic nerve. Mechanical allodynia after CCI in the lesioned hind paw (A) or mechanical threshold in the sham-operated hind paw (B) was assessed in Wistar 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 five 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 were assessed each day 4 h after TRO19622 and vehicle treatment or 1 h after gabapentin dosing. Values are means \pm S.E.M. ($n = 6/\text{group}$). †, $p < 0.001$ versus BL values; ***, $p < 0.001$ compared with the vehicle group.

is interesting to note that TRO19622 plasma concentrations in STZ-diabetic rats were approximately 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. Because 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 seems to be below the level needed for efficacy.

Likewise, a single administration of 10 mg/kg, which results in a similar plasma concentration ($\sim 0.7 \mu\text{M}$; 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

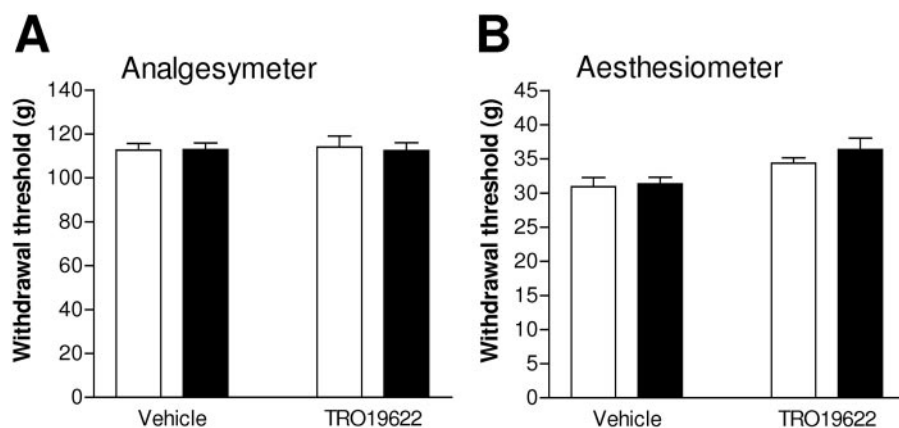


Fig. 5. Acute administration of TRO19622 did not reverse mechanical allodynia and hyperalgesia in naive rats. Behavioral thresholds to mechanical stimulation were assessed in Wistar rats before treatment (white columns) and 4 h after (black columns) oral administration of vehicle (corn oil; 5 ml/kg) or TRO19622 (100 mg/kg) using a dynamic plantar aesthesiometer and an analgesimeter (Ugo Basile) as described under *Materials and Methods*. Values are means \pm S.E.M. ($n = 7-8$ /group).

TABLE 3

Responses in the formalin test

Test substances were administered orally to groups of six adult Wistar rats 60 min (morphine) or 4 h (TRO19622 or vehicle) before intraplantar injection of formalin (0.05 ml; 5% solution). The paw licking time was recorded in 5-min intervals; 0 to 5 min corresponded to the first phase and 15 to 30 min corresponded to the second phase. Data shown are mean \pm S.E.M. of the licking time (seconds) and as percentage of reduction compared with vehicle-treated group.

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

** $p < 0.01$; *** $p < 0.001$ versus vehicle-treated animals.

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 4 h after the last administration of TRO19622. Values are means \pm S.E.M. ($n = 3-6$ rats).

Study	TRO19622 Treatment		TRO19622 Plasma Conc.	
	Treatment Duration	Dose	μ M	μ g/ml
		mg/kg		
STZ (nerve conduction study)	32 days	3	0.8 \pm 0.3	0.3 \pm 0.1
		30	8.4 \pm 3.3	3.3 \pm 1.3
		300	>50	>20
STZ (pain study)	5 days	10	4.5 \pm 1.8	1.8 \pm 0.7
		30	8.1 \pm 2.4	3.3 \pm 0.9
		100	37.5 \pm 7.3	15.0 \pm 2.9
Vincristine	5 days	10	2 \pm 1.3	0.8 \pm 0.5
		30	3.9 \pm 1.1	1.6 \pm 0.4
		100	14.2 \pm 1.4	5.7 \pm 0.6
CCI	5 days	3	0.5 \pm 0.2	0.2 \pm 0.1
		30	4.2 \pm 1.5	1.7 \pm 0.6
		300	24.3 \pm 0.5	9.7 \pm 2.0
Formalin Pharmacokinetics	Acute	300	5.5 \pm 2.1	2.2 \pm 0.8
		10	0.7 \pm 0.2	0.3 \pm 0.1
		100	8.6 \pm 1.2	3.4 \pm 0.5

concentrations between 14.2 and 37.5 μ M 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 μ M, 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 antinociceptive 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. Likewise, 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 they persisted after chronic administration. It is interesting to note that TRO19622 did not have analgesic activity in models of acute noxious pain or in models with inflammatory components, such as formalin-induced pain or chronic constriction nerve injury.

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 by 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 normoglycemia. It is interesting to note that these deficits do not correlate with any structural impairment in axonal diameter or in myelin sheath; rather, they are 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. It is interesting to note that improvement in motor nerve conduction in rats receiving STZ injections could be detected with chronic oral administration of doses as low as 3 mg/kg/day producing steady-state plasma concentrations of $\sim 0.8 \mu\text{M}$ (Table 4), which is similar to that found with the lowest active dose of TRO19622 shown to accelerate nerve recovery in mice after a sciatic nerve crush (Bordet et al., 2007). TRO19622 did not modify hyperglycemia or increase body weight in rats receiving STZ injections, excluding a general improvement in the condition of the animals 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 reported previously (Hanaoka et al., 1992, 1994). As suggested by these findings, diabetic rats showed significant reduction in NGF and neurotrophin-3 in sciatic nerve and in innervated skin and muscle (Hellweg et al., 1994; Fernyhough et al., 1998). It is interesting to note that exogenous administration of both NGF and neurotrophin-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. It is interesting to note that TRO19622 was found to reverse both thermal and established tactile allodynia after the first oral administration, with efficacy comparable with that of morphine or gabapentin. This acute antinociceptive effect was dose-dependent with a single administration and significant at the highest dose, whereas 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. It is interesting to note that

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 seem 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 seems 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 anticonvulsant activity even after repeated administration of up to 300 mg/kg/day for 5 days in mice (supplemental data), and as reported here, it did not demonstrate analgesic activity in pain models in which anticonvulsants, opiates, and local anesthetics are active. At doses up to 300 mg/kg/day, 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 naive 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 antinociceptive effects of TRO19622 have different mechanism of action compared with current anticonvulsant, antidepressant, anesthetic or analgesic treatments and that this mechanism may distinguish syndromes such as 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-dependent 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; Leininger et al., 2006; Siau and Bennett, 2006). Increased production of mitochondrial reactive oxygen species 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 (Leininger 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-fibers and myelinated axons, whereas there was no evidence of nerve degeneration or 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. Likewise, peripheral nerve biopsies from patients suffering from vincristine-evoked painful neuropathy revealed axonal and mitochondrial swelling, whereas microtubule alterations were not evidenced (Thant et al., 1982).

Mitochondrial abnormalities have recently been linked to dysregulation of intracellular calcium homeostasis in the context of neuropathic pain. Intrathecal administration of

calcium chelators significantly inhibited mechanoallodynia and mechanohyperalgesia in rats treated with antihuman 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 and CCI 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 after injury; Kleinschnitz et al., 2004) after 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 β -tubulin and the mPTP protein complex have been shown (Carré et al., 2002). Acetyl-L-carnitine (ALCAR) facilitates β -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, whereas 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 voltage-dependent anion channel and peripheral benzodiazepine receptor 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, whereas 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 seems 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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Address correspondence to: Dr. Rebecca M. Pruss, Trophos, Parc Scientifique de Luminy, Luminy Biotech Entreprises, Case 931, 13288 Marseille Cedex 9, France. E-mail: rpruss@trophos.com
