Published in final edited form as:
Pain. 2009 December 15; 147(1-3): 202–209. doi:10.1016/j.pain.2009.09.006.

Olesoxime (cholest-4-en-3-one, oxime): Analgesic and neuroprotective effects in a rat model of painful peripheral neuropathy produced by the chemotherapeutic agent, paclitaxel

Wen Hua Xiao[a,b], **Felix Y. Zheng**[a], **Gary J. Bennett**[a,b,c], **Thierry Bordet**[d], and **Rebecca M. Pruss**[d]

[a] Department of Anesthesia, McGill University, Montreal, Quebec, Canada

[b] The Alan Edwards Centre for Research on Pain, McGill University, Montreal, Quebec, Canada

[c] Faculty of Dentistry, McGill University, Montreal, Quebec, Canada

[d] Trophos SA, Marseille, France

Abstract

Olesoxime is a small cholesterol-like molecule that was discovered in a screening program aimed at finding treatment for amyotrophic lateral sclerosis and other diseases where motor neurons degenerate. In addition to its neuroprotective and pro-regenerative effects on motor neurons *in vitro* and *in vivo*, it has been shown to have analgesic effects in rat models of painful peripheral neuropathy due to vincristine and diabetes. We used a rat model of painful peripheral neuropathy produced by the chemotherapeutic agent, paclitaxel, to determine whether olesoxime could reverse established neuropathic pain. In addition, we determined whether giving olesoxime during the exposure to paclitaxel could prevent the development of the neuropathic pain syndrome and the accompanying degeneration of the terminal arbors of sensory fibers in the epidermis. Olesoxime significantly reduced established mechano-allodynia and mechano-hyperalgesia. There was no indication of tolerance to the effect during five days of dosing and the analgesia persisted for 5-10 days after the last injection. Giving olesoxime during the exposure to paclitaxel significantly and permanently reduced the severity of mechano-allodynia and mechano-hyperalgesia and significantly reduced the amount of sensory terminal arbor degeneration. Olesoxime targets mitochondrial proteins and its effects are consistent with the mitotoxicity hypothesis for paclitaxel-evoked painful peripheral neuropathy. We conclude that olesoxime may be useful clinically for both the prevention and treatment of paclitaxel-evoked painful peripheral neuropathy.

Keywords

chemotherapy; mitochondria; neuropathic pain; neuroprotection; TSPO; VDAC

Correspondence: Wen Hua Xiao, MD McIntyre Bldg., Room 1213 3655 Promenade Sir William Osler Montreal, Quebec Canada H3G 1Y6 **Telephone:** 514.398.1263 **FAX:** 514.398.8241 wenhua.xiao@mcgill.ca.

1. Introduction

Olesoxime (cholest-4-en-3-one, oxime; TRO19622) is a small (molecular weight: 399) cholesterol-like compound with neuroprotective, pro-regenerative, and analgesic actions. *In vitro*, olesoxime promotes survival and stimulates neurite outgrowth of motor neurons cultured in the absence of neurotrophic factors [2]. *In vivo*, olesoxime improves motor performance and increases survival in a mouse model of familial amyotrophic lateral sclerosis [2]. It also promotes axonal regeneration after sciatic nerve crush in adult mice, prevents the death of motor neurons following axotomy in neonatal rats, and improves motor nerve conduction velocity deficits in diabetic rats [2,3]. In addition, olesoxime reverses mechano-allodynia in rats with painful peripheral neuropathy due to vincristine treatment or streptozotocin-induced diabetes [3]. However, olesoxime has no effect on pain sensitivity in normal animals, no effect in the CCI model of post-traumatic painful peripheral neuropathy, and no effect on Phase I or II responses in the formalin test. It is not an anti-convulsant or a sedative [3].

Work with an animal model of the painful peripheral neuropathy produced by the chemotherapeutic agent, paclitaxel, has shown that the condition is characterized by an increased incidence of swollen and vacuolated axonal mitochondria, degeneration of the intraepidermal terminal arbors of sensory axons, and an abnormal spontaneous discharge in sensory afferents. It has been hypothesized that the degeneration, abnormal discharge, and pain are all secondary to paclitaxel-evoked dysfunction in axonal mitochondria [9-11,21,22,26].

Paclitaxel is known to have direct effects on mitochondria. It increases conductance through the mitochondrial permeability transition pore (mPTP), a channel that spans the mitochondrial inner membrane and opens when mitochondria are damaged by excessive calcium, ADP and ATP depletion, and oxidative stress [1,7,12,15]. mPTP opening is involved in both necrotic and apoptotic cell death. Paclitaxel's effect on mPTP may be mediated through its binding to mitochondrial β-tubulin, which closely associates with and modulates opening of the voltage-dependent anion channel (VDAC) [4,19,20]. VDAC, known also as porin, regulates the passage of Ca^{2+}, ADP and ATP, Kreb's cycle substrates, and low molecular weight substances across the mitochondrial outer membrane. Changes in VDAC conductance modulate mPTP opening [15].

Olesoxime is known to bind to VDAC as well as to the mitochondrial translocator protein (18kDa) (TSPO) another outer mitochondrial membrane protein implicated in mPTP modulation [2,16,17]. In neurons undergoing apoptosis, olesoxime reduces cytochrome c release from mitochondria, demonstrating an inhibitory effect on mPTP [2]. Thus, if paclitaxel-evoked neurotoxicity involves mPTP-mediated and/or VDAC-mediated mitochondrial dysfunction, then olesoxime may be beneficial.

Using a rat model of paclitaxel-evoked painful peripheral neuropathy, we investigated whether olesoxime could reverse established neuropathic pain and whether it could prevent the development of the paclitaxel-evoked pain syndrome and the associated degeneration of sensory terminal arbors in the epidermis.

2. Methods

All experimental protocols were approved by the Animal Care Committee of the Faculty of Medicine, McGill University, and were conducted in accordance with the ethics guidelines of the International Association for the Study of Pain, the National Institutes of Health (USA), the Canadian Institutes of Health Research, and the Canadian Council on Animal Care.

2.1. Animals

Adult male Sprague-Dawley rats (200-300 g, Harlan Inc., Indianapolis, IN; Frederick, MD breeding colony) were housed on sawdust bedding in plastic cages with food and water available *ad libitum* and lighting on a fixed 12 h light-dark cycle.

2.2. Drug administration

Paclitaxel was prepared fresh daily by diluting Taxol®, (Bristol-Myers-Squibb: 6 mg/ml paclitaxel in Cremophor/EL vehicle) with saline to a final concentration of 2 mg/ml. Rats were given intraperitoneal injections of paclitaxel (2 mg/kg) on 4 alternate days (day 0 (D0), D2, D4 and D6) as described previously [18].

Olesoxime (Trophos SA, Marseille, France) was prepared fresh daily in corn oil (Sigma, St. Louis, MA). Olesoxime or the vehicle was administered via oral gavage in a volume of 5.0 ml/kg. The olesoxime doses used here (3-100 mg/kg) were chosen based on prior reports of neuroprotective and analgesic activity [2,3].

2.3. Behavioral testing

The rats were habituated to the behavioral testing environment and two baseline tests of mechanical sensitivity were conducted as previously described [8,9]. von Frey filaments with bending forces of 4 g and 15 g were applied to the mid-plantar skin of each hind paw 5 times, with each application held for 5 seconds. Withdrawal responses from both hind paws were counted and then expressed as an overall percentage response. The increased level of respond to the 4 g stimulus seen after paclitaxel treatment is indicative of mechano-allodynia because normal rats rarely, if ever, withdraw from this stimulus. In normal rats the 15 g stimulus evokes a withdrawal response 15–20% of the time, indicating that this is a mildly noxious stimulus [8,9]; thus, the increased level of respond seen after paclitaxel treatment is indicative of mechano-hyperalgesia.

2.4. Treatment paradigm

To determine whether olesoxime has an analgesic effect on established paclitaxel-evoked pain, we examined withdrawal responses in animals after daily oral dosing with olesoxime during the period of approximate peak pain severity. Baseline responses in the behavioral tests were done on D23 and D24 after the first administration of paclitaxel (the approximate beginning of the plateau phase of maximal pain severity), and three experimental groups were formed such that each had approximately equal mechano-allodynia and mechano-hyperalgesia. The groups (each n = 12) were then randomly assigned to receive olesoxime (10 mg/kg or 100 mg/kg) or vehicle on 5 consecutive days, beginning on D27. Behavior was tested 4 h after each of the daily administrations. Behavior was also assessed during a washout period beginning 1 day after the last administration of olesoxime/vehicle (washout day 1; WD1), and on WD3, WD5, WD10, WD14, and WD18. Behavioral assays were done by an observer who was blind as to group assignment.

2.5. Preventive paradigm

To determine whether olesoxime could prevent the development of paclitaxel-evoked painful peripheral neuropathy, three experimental groups were compared (each n = 12). The groups were administered vehicle or olesoxime at 3 mg/kg or 30 mg/kg daily for 17 consecutive days, starting the day prior to the first injection of paclitaxel ($D^{-}1$) until 9 days after the last injection of paclitaxel (D15). Dosing was continued after the last paclitaxel injection because there is a delay of several days before the onset of statistically significant pain hypersensitivity [8] and the time of onset of the pain-producing pathology is thus uncertain. On those days when both drugs were to be administered, olesoxime was given at 0900 h and paclitaxel at 1300 h.

Behavioral assays were repeated every 3-5 days beginning on D16 until D40 by an observer who was blind as to group assignment.

2.6. Effects of prophylactic treatment on paclitaxel-evoked intraepidermal nerve fiber degeneration

The paclitaxel model used here has been shown to be associated with a significant loss of intraepidermal nerve fibers (IENFs), i.e., the sensory terminal receptor arbors of the afferents that innervate the epidermis [11,22]. To determine whether olesoxime prevents this degeneration, the prophylactic dosing protocol described above was repeated in three groups of rats (olesoxime at 3 mg/kg or 30 mg/kg, or vehicle; each n = 12). Behavioral tests were done on D29 and D30 to confirm the presence of the expected paclitaxel-evoked pain hypersensitivity in the vehicle-treated group and the expected analgesic effects in the 3 mg/kg and 30 mg/kg groups. On D31, eight rats were randomly selected from each group and sacrificed for the immunocytochemical assessment of IENFs. An additional four naïve rats (i.e., neither paclitaxel nor olesoxime treatment) of the same age and weight were sacrificed as normal controls.

Our procedures for the immunocytochemical detection and quantification of IENFs are described in detail elsewhere [11,22]. Briefly, the rat was perfused with paraformaldehyde and hind paw glabrous skin was harvested from the heel region (that part of the paw that lies proximal to the plantar tori and distal to the calcaneous). The full-thickness skin samples were sectioned at 30 μm on a cryostat. The pan-neuronal marker, PGP9.5, was visualized with a well characterized primary antibody (Research Diagnostics; Flanders, NJ) diluted 1:6400 and a Cy3-labeled secondary antibody (Jackson ImmunoReseach) diluted 1:200.

IENF counts were done by an observer blind as to the animal's group assignment. Using a 40X objective, all ascending nerve fibers that were seen to cross into the epidermis were counted; no minimum length was required, and fibers that branched within the epidermis were counted as one. A low magnification montage of each section was made and the length of the epidermal border was measured. The IENF counts were expressed as the number per cm of epidermal border. One section of skin was analyzed for each rat in each group. The average section length was 11.4 mm (range: 8.8-13.0 mm) and thus the amount of tissue sampled per animal met or exceeded clinical guidelines for IENF quantification [13].

2.7. Effects on paclitaxel-evoked spontaneous discharge

Paclitaxel-evoked painful peripheral neuropathy is associated with an abnormal incidence of spontaneously discharging A-fibers and C-fibers [26]. To determine whether the acute analgesic effects of olesoxime were associated with suppression of this discharge, we surveyed the incidence of spontaneously discharging fibers in rats that had been treated with vehicle or 100 mg/kg olesoxime (each n = 6) on two consecutive days (the treatment paradigm study described above found significant anti-allodynic and anti-hyperalgesic effects after this treatment). All rats had confirmed paclitaxel-evoked pain (assessed on D23-D24) and subsequently received olesoxime or vehicle treatment during the plateau phase of peak pain severity (D27-D44). Electrophysiological experiments began on the second day of treatment, 4 h after drug/vehicle administration. The paclitaxel-treated rats were compared to a group of four naïve rats (neither paclitaxel nor olesoxime exposure). The experimenter was blind as to the rat's group assignment.

Surgical preparation for fiber recordings required about 1 h and data were collected over the next 2-3 h when plasma concentrations of olesoxime are maximal after oral administration. Recording methods were identical to those described previously [25-27]. Briefly, the number of individually-identifiable fibers in each microfilament was determined and the incidence of

individually-identifiable fibers with spontaneous discharge was noted, as was their discharge frequency. The conduction velocity was determined for all individually-identifiable fibers. We did not differentiate between A-fibers with conduction velocities in the A□ and A□ ranges because it is impossible to differentiate functional classes of A-fibers on this basis [6]. We purposely avoided characterizing the fibers' responses to receptive field stimulation. To do so would require repeated application of noxious stimuli that might sensitize nociceptors. Sensitized nociceptors have an ongoing discharge that would be impossible to distinguish from paclitaxel-evoked spontaneous discharge.

2.8. Olesoxime plasma levels

Blood was drawn from the tail vein in animals enrolled in the behavioral studies or via cardiac puncture in the animals sacrificed for the anatomical and electrophysiological studies, collected in lithium-heparin tubes, centrifuged at 3000 rpm for 10 min and the plasma frozen on Dry Ice. Quantification was performed via high-performance liquid chromatography with MS/MS detection. The detection limit of the assay was 0.01 μM.

3. Results

3.1. Treatment paradigm

As expected, paclitaxel evoked statistically significant mechano-allodynia and mechano-hyperalgesia when assessed on D23 and D24 (Fig. 1). Also as expected, paclitaxel-treated rats that received only vehicle continued to have significant mechano-allodynia and mechano-hyperalgesia for the duration of the study (D47).

3.1.1. Olesoxime reversal of established mechano-hyperalgesia—Both doses of olesoxime significantly suppressed mechano-hyperalgesia after the 1st administration and continued to produce the same effect after the 2nd-5th administrations (Fig. 1). There was no indication of the development of tolerance to the anti-hyperalgesic effect. The average reduction in hyperalgesia after the 1st-5th administration was 52% for the 10 mg/kg dose and 59% for the 100 mg/kg dose. For all time points during the daily dosing period, the reductions in mechano-hyperalgesia were partial, i.e. the response rates were still significantly elevated relative to the baseline (pre-paclitaxel) response rate (all at least $p < 0.05$; repeated measures ANOVA followed by Dunnett"s t-test). For both doses, the anti-hyperalgesic effect persisted until at least the 5th day after olesoxime administration. There were no statistically significant differences between the anti-hyperalgesic effects of the 10 mg/kg and 100 mg/kg doses at any time point.

3.1.2. Olesoxime reversal of established mechano-allodynia—Neither the 10 mg/kg nor 100 mg/kg dose had any significant effect on mechano-allodynia when tested 4 h after the 1st administration. However, both doses produced a statistically significant reversal of mechano-allodynia after the 2nd -5th administrations (Fig. 1). The average reduction in allodynia after the 2nd-5th administrations was 54% for the 10 mg/kg dose and 58% for the 100 mg/kg dose. No tolerance to the anti-allodynic effect was seen. For the 2nd-5th administrations, the reductions in mechano-allodynia were partial, i.e. the response rates were still significantly elevated relative to the baseline (pre-paclitaxel) response rate (all at least $p < 0.05$; repeated measures ANOVA followed by Dunnett"s t-test). For both doses, the anti-allodynic effect persisted until at least the 10th day after olesoxime administration (WD10). The anti-allodynic effects of the 10 mg/kg and 100 mg/kg doses were not significantly different at any time point.

3.1.3. Olesoxime plasma levels—For the 10 mg/kg dose, olesoxime plasma levels (mean ± SD) were 0.82 ± 0.13 □M after the 1st administration and 1.39 ± 0.18 □□ after the 5th

administration. For the 100 mg/kg dose, the corresponding levels were 6.75 ± 2.2 □□ and 8.91 ± 0.56 □□.

3.2. Preventive paradigm

Prior to treatment, there were no significant differences in the pre-paclitaxel baseline response frequencies in the three groups randomly assigned to receive vehicle or the two doses of olesoxime. Vehicle-treated animals developed the expected mechano-allodynia and mechano-hyperalgesia following paclitaxel treatment; when compared to their own baseline responses, statistically significant mechano-allodynia and mechano-hyperalgesia were present as early as D16 and persisted for the duration of the study (Fig. 2).

3.2.1. Effects of olesoxime on paclitaxel-induced allodynia and hyperalgesia— Both the 3 mg/kg and 30 mg/kg olesoxime doses significantly and permanently reduced the severity of mechano-allodynia and mechano-hyperalgesia from D16 until the end of the study on D40 (Fig. 2). The magnitude of the effects of the two doses was nearly identical at all time points. The average reduction in the severity of mechano-allodynia and mechano-hyperalgesia for the period D16-D40 was, respectively, 62% and 54% for the 3 mg/kg dose, and 54% and 46% for the 30 mg/kg dose.

3.2.2 Olesoxime plasma levels—For the 3 mg/kg dose, the olesoxime plasma level (mean ± SD) sampled 4 h after the 15th dose was 0.17 ± 0.06 □□; for the 30 mg/kg dose the corresponding level was 2.69 ± 0.2 □□. No olesoxime was detected in plasma taken on D25 or D40 in either group.

3.3. Effects of olesoxime on IENF degeneration

IENFs were easily identified in PGP9.5-stained sections (Fig. 3). Punctate immunoreactivity in the vital layers of the epidermis provided a clear demarcation with the underlying dermis. No staining was observed in sections prepared without exposure to the primary antisera.

3.3.1. Behavioral confirmation of the effects of olesoxime—The rats used in this anatomical study were first assayed behaviorally to confirm the presence of paclitaxel-evoked pain and the analgesic effect of preventive dosing with olesoxime. Paclitaxel-treated rats given vehicle developed the expected statistically significant mechano-allodynia and mechano-hyperalgesia on D29-D30 (Fig. 4A). Prophylactic treatment with both the 3 mg/kg and 30 mg/kg doses of olesoxime significantly decreased the severity of mechano-allodynia and mechano-hyperalgesia. There were no significant differences between the magnitudes of the analgesic effects of the two doses. The magnitude of the suppression of mechano-allodynia and mechano-hyperalgesia on D29-D30 was comparable to that seen in the previous experiment (Fig. 2).

3.3.2. IENF counts—The naïve control rats had 317 ± 7.6 (mean ± SEM) IENFs per cm of epidermal border (Fig. 4B). This value is similar to the normal values that we found in prior studies [17,28].

As expected, paclitaxel-treated rats that received vehicle had a statistically significant reduction in IENFs: 172 ± 14.0 per cm; a reduction of 46% relative to the naïve control group.

Paclitaxel-treated rats that received prophylactic treatment with 3 mg/kg/d or 30 mg/kg/d olesoxime had 239 ± 17.6 and 247 ± 14.4 IENFs per cm, respectively. For both doses, the decreases are significantly less than the 46% decrease seen in the paclitaxel-treated rats administered vehicle. However, both doses produced decreases (25% and 22%) that are significantly different relative to the naïve control group. Thus, the neuroprotective effect was significant but partial.

3.3.3. Olesoxime plasma levels—Olesoxime levels sampled 4 h after the 15th dose were 0.39 ± 0.04 □□ in the 3 mg/kg group and 2.9 ± 0.86 □□ in the 30 mg/kg group. These values are similar to those of the prior study.

3.4. Effects of olesoxime on paclitaxel-evoked spontaneous discharge

3.4.1. Recording from naïve controls—We sampled 261 A-fibers and 70 C-fibers in 34 microfilaments from naïve control rats. Spontaneous discharge was found in 1.6% of the A-fibers and 0% of the C-fibers (Fig. 5). These low levels of spontaneous discharge in normal rats are nearly identical to what we have found in previous studies [25-27].

3.4.2. Recording from vehicle-dosed paclitaxel-treated rats—We sampled 238 A-fibers and 97 C-fibers in 51 microfilaments from paclitaxel-treated rats that received two consecutive daily doses of vehicle. Spontaneous discharge was found in 14.2% of the A-fibers and 25.2% of the C-fibers (Fig. 5). The increase in the incidence of spontaneous discharge was statistically significant relative to the naïve control group for both A-fibers and C-fibers ($p < 0.01$, χ^2 test). The average discharge frequency of the spontaneously active A-fibers was 1.3 ± 0.06 Hz (mean ± SEM) and 1.2± 0.03 Hz in the C-fibers. Both A-fibers and C-fibers had an irregular pattern of discharge. The incidence, discharge frequency, and discharge pattern in the A-fibers and C-fibers are very similar to what we found in a previous study of paclitaxel-treated rats [26].

3.4.3. Recording from paclitaxel-treated rats dosed with olesoxime—We sampled 228 A-fibers and 105 C-fibers in 53 microfilaments from paclitaxel-treated rats that had received two consecutive daily doses of 100 mg/kg olesoxime. This treatment was previously shown to significantly reduce both allodynia and hyperalgesia (Fig. 1). Recording occurred during a time period corresponding to the peak plasma concentration following oral administration. Spontaneous discharge was found in 13.3% of the A-fibers and 23.3% of the C-fibers (Fig. 5). The average discharge frequency of the spontaneously active A-fibers was 1.1 ± 0.07 Hz (mean ± SEM) and 1.1 ± 0.03 Hz in the C-fibers, and both fiber types had an irregular pattern of discharge. There were no significant differences between the vehicle and olesoxime groups for the incidence of spontaneous discharge or its frequency for either A-fibers or C-fibers.

3.4.4. Olesoxime plasma levels—The plasma level of olesoxime sampled at the conclusion of the recording session was 9.31 ± 2.05 □□, which is close to the level found after the 5th dose of 100 mg/kg seen in the treatment paradigm experiment described above.

4. Discussion

These experiments yielded three new observations, each of which has potential clinical importance: (1) Olesoxime given prophylactically significantly reduces paclitaxel-evoked neuropathic pain. (2) Prophylactic administration of olesoxime also significantly reduces paclitaxel-evoked neurodegeneration. (3) A short (five days) course of olesoxime given when paclitaxel-evoked neuropathic pain is established produces persistent anti-allodynia and anti-hyperalgesia.

4.1. Analgesic effects of olesoxime

We found that a single administration of 10 mg/kg olesoxime had an anti-hyperalgesic effect on established paclitaxel-evoked neuropathy. An anti-allodynic effect was found after a second administration of 10 mg/kg. Analgesic action with of a single oral administration of olesoxime (100 mg/kg) has also been seen in animals with neuropathic pain due to vincristine or streptozotocin-induced diabetes [3].

Five daily administrations of olesoxime had a persistent analgesic effect; the anti-hyperalgesic effect lasted for at least five days after dosing and the anti-allodynic effect lasted for at least 10 days. It is possible that the mechanism of action for this persistent effect is different from that which underlies the acute analgesic effect and may instead be related to the mechanism that accounts for olesoxime's ability to inhibit the development of paclitaxel-evoked pain and IENF degeneration (see below). Alternatively, the site of action responsible for acute analgesic effects of olesoxime may accumulate higher concentrations and have a slower rate of washout than plasma. As there were no changes in the degree of pain relief seen with the 2^{nd}-5^{th} daily treatments, it appears that tolerance does not develop to olesoxime's analgesic effect.

These results suggest that olesoxime may be useful clinically in the treatment of established chemotherapy-evoked neuropathic pain. Other potential analgesics for chemotherapy-evoked neuropathic pain almost certainly have different mechanisms of action [28], suggesting the possible usefulness of combination therapy with olesoxime.

4.2. Prophylactic effects of olesoxime on paclitaxel-induced neuropathy

The prophylactic administration of olesoxime had a significant inhibitory effect on the development of paclitaxel-evoked mechano-allodynia and mechano-hyperalgesia. The effect was seen with a steady-state plasma level (D15) of 0.17 □M. The effect appears to represent a true preventive action because it persisted for the duration of the experiment (until D40, 25 days after the last dose) and during this period (D25-D40) no olesoxime could be detected in plasma.

We find that prophylactic dosing with 3 mg/kg reduced paclitaxel-evoked degeneration of IENFs by 50%. This is the first demonstration that olesoxime has a neuroprotective effect against a chemotherapy-evoked nerve injury. Previous experiments have shown that olesoxime has neuroprotective and pro-regenerative effects on motor neurons [2]. IENFs are the terminal receptor arbors of primary afferent neurons and our data thus indicate that olesoxime is neuroprotective for both sensory and motor neurons.

These results suggest that olesoxime treatment may be clinically useful as a prophylactic treatment for both paclitaxel-evoked neuropathy and the neuropathic pain syndrome that sometimes accompanies it. The feasibility of such use is supported by preliminary results showing that olesoxime does not interfere with the anti-mitotic or cytotoxic effects of paclitaxel or vincristine on proliferating tumor cell lines (Braguer et al., manuscript in preparation).

4.3. Mechanisms of action

No interactions have been found between olesoxime and any ion channels or neurotransmitter receptors [2]. Olesoxime treatment (100-300 mg/kg) has no effect on the pain responses of normal animals and no effect on Phase I responses in the formalin test. Significantly, olesoxime (5 daily doses of 300 mg/kg) is without effect in the CCI model of post-traumatic painful peripheral neuropathy and administration of 300 mg/kg has no effect on Phase II responses in the formalin test. It is thus clear that olesoxime has a unique analgesic mechanism of action [2,3]. Olesoxime has been documented to bind to two outer mitochondrial membrane proteins: TPSO and VDAC [2]. Mitochondrial pathology is known to accompany chemotherapy and diabetic neuropathies [9,14]. Thus, it is most likely that the effects on paclitaxel-evoked peripheral neuropathy that we demonstrate here are due to a mitochondrial mechanism of action.

We show here that olesoxime at 10 mg/kg and 100 mg/kg has an acute analgesic effect. We found no significant difference between the magnitudes of the analgesia produced by these two doses although the full activity with the 10 mg/kg dose required repeated administration. In

the studies of Bordet et al. [3] the threshold plasma concentration for the acute analgesic effect of olesoxime required plasma concentrations >1 □M, which was obtained with a single administration of 100 mg/kg or repeated administration of 10 mg/kg; a dose of 3 mg/kg was too low to reverse pain behavior in diabetic rats even with repeated administration. Therefore, the apparent lack of dose effect in the studies reported here is likely due to the fact that both 10 and 100 mg/kg are above the minimally effective dose required for maximal efficacy. In our prophylactic dosing studies (Figs. 2,4), we found no differences in the size of the analgesic (or neuroprotective) effects of olesoxime at 3 mg/kg and 30 mg/kg. The absence of a dose-response relationship for these effects is puzzling. The minimally active dose for neuroprotective effects in motor neurons appears to be between 0.3 and 3 mg/kg/d [2,3]. In any case, it seems certain that we have not underestimated the maximum achievable effect because of inadequate dosing.

The protective effect seen with 3 mg/kg in the prophylactic dosing paradigm was achieved with a steady-state plasma level that was 5-10 times lower than the estimated [3] threshold levels (≥1 μM) for the acute analgesic effect. Thus, we think it is possible that different mechanisms may be responsible for olesoxime's acute analgesic effect and its preventive effect on the development of neuropathic pain and IENF degeneration.

4.3.1. Mechanisms for acute analgesia—A single administration of olesoxime reduces established neuropathic pain. The mechanism for this acute analgesic effect is obscure. We found no evidence that the effect is due to an acute suppression of paclitaxel-evoked spontaneous discharge. As the analgesia is apparent within 4 h after a single oral administration, it seems very unlikely that it can be related to reversal of paclitaxel-evoked IENF degeneration. Cata et al. [5] have shown increased levels of excitability in spinal cord dorsal horn neurons in rats with paclitaxel-evoked neuropathic pain. It is conceivable that an olesoxime effect on the mitochondria within spinal cord neurons and synapses might reverse this hyperexcitability and thereby reverse allodynia and hyperalgesia.

4.3.2. Mechanisms for protective effects—Prophylactic treatment with olesoxime significantly reduced the development of paclitaxel-evoked pain and IENF degeneration. Both of these effects were partial and both were reduced by about 50%.

We have shown that paclitaxel causes mitochondria to swell and become vacuolated and we have hypothesized that these structural alterations are signs of functional impairment [9-11, 21,22,26]. Recent data show that mitochondria in peripheral nerves from paclitaxel-treated rats are indeed functionally impaired: they have a reduced capacity for oxidative phosphorylation and signs of a high level of oxidative stress (Zheng, Xiao, and Bennett, unpublished data). Mitochondrial dysfunction is expected to lead to a deficiency in the axon's energy supply and this would be expected to result in degeneration once a certain threshold was reached. The threshold would be expected to vary with energy requirement and the IENF is likely to be a region of high energy demand. Thus it is possible that the ability of olesoxime to reduce paclitaxel-evoked IENF degeneration is due to its interaction with mitochondria.

The prophylactic effect of olesoxime on the development of mechano-allodynia and mechano-hyperalgesia may be related to its prevention of IENF degeneration. Degenerating fibers may give rise to spontaneous discharge and the products of degeneration may evoke discharge in intact neighbouring fibers [24]. Moreover, an energy deficit in IENFs might result in membrane depolarization and the generation of spontaneous discharge in fibers that have not degenerated or are in an early stage of degeneration. Although these assumptions predict that olesoxime ought to reduce the abnormal spontaneous discharge that has been shown to occur after paclitaxel treatment, we found no evidence of the suppression of spontaneous discharge in sensory peripheral afferent A- or C-fibers following acute exposure to olesoxime. Additional

experiments will be needed to determine whether prophylactic treatment that inhibits IENF degeneration suppresses the development of spontaneous discharge.

Acknowledgments

This work was supported by grants from Trophos SA, the National Institute of Neurological Disorders and Stroke (R01 NS052255), and the Canada Foundation for Infrastructure. T.B. and R.M.P. are employees of Trophos SA. G.J.B. is a Canada Senior Research Chair. We thank the analytical chemistry team at Trophos SA for plasma olesoxime analysis, and D. Braguer and M. Carré for sharing unpublished data.

References

1. Andre N, Braguer D, Brasseur G, Goncalves A, Lemesle-Meunier D, Guise S, Jordan MA, Briand C. Paclitaxel induces release of cytochrome c from mitochondria isolated from human neuroblastoma cells. Cancer Res 2000;60:5349–53. [PubMed: 11034069]
2. 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, Pruss RM. Identification and characterization of cholest-4-en-3-one, oxime (TRO19622), a novel drug candidate for amyotrophic lateral sclerosis. J Pharmacol Exp Ther 2007;322:709–20. [PubMed: 17496168]
3. Bordet T, Buisson B, Michaud M, Abitbol JL, Marchand F, Grist J, Andriambeloson E, Malcangio M, Pruss RM. Specific anti-nociceptive activity of cholest-4-en-3-one, oxime (TRO19622) in experimental models of painful diabetic and chemotherapy-induced neuropathy. J Pharmacol Exp Ther 2008;326:623–32. [PubMed: 18492948]
4. Carrè M, Andre N, Carles G, Borghi H, Brichese L, Briand C, Braguer D. Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel. J Biol Chem 2002;277:33664–9. [PubMed: 12087096]
5. Cata JP, Weng HR, Chen JH, Dougherty PM. Altered discharges of spinal wide dynamic range neurons and down-regulation of glutamate transporter expression in rats with paclitaxel-induced hyperalgesia. Neuroscience 2006;138:329–38. [PubMed: 16361064]
6. Djouhri L, Lawson SN. A-beta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res Rev 2004;46:131–45. [PubMed: 15464202]
7. Evtodienko YV, Teplova VV, Sidash SS, Ichas F, Mazat JP. Microtubule-active drugs suppress the closure of the permeability transition pore in tumour mitochondria. FEBS Lett 1996;393:86–8. [PubMed: 8804430]
8. Flatters SJL, Bennett GJ. Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. Pain 2004;109:150–61. [PubMed: 15082137]
9. Flatters SJL, Bennett GJ. Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: Evidence for mitochondrial dysfunction. Pain 2006;122:247–57.
10. Flatters SJL, Xiao WH, Bennett GJ. Acetyl-L-carnitine prevents and reduces paclitaxel-induced painful neuropathy. Neurosci Lett 2006;397:219–23. [PubMed: 16406309]
11. Jin HW, Flatters SJL, Xiao WH, Mulhern HL, Bennett GJ. Prevention of paclitaxel-evoked painful peripheral neuropathy by acetyl-L-carnitine: Effects on axonal mitochondria, sensory nerve fiber terminal arbors, and cutaneous Langerhans cells. Exptl Neurol 2008;210:229–37. [PubMed: 18078936]
12. Kidd JF, Pilkington MF, Schell MJ, Fogarty KE, Skepper JN, Taylor CW, Thorn P. Paclitaxel affects cytosolic calcium signals by opening the mitochondrial permeability transition pore. J Biol Chem 2002;277:6504–10. [PubMed: 11724773]
13. Lauria G, Cornblath DR, Johansson O, McArthur JC, Mellgren SI, Nolano M, Rosenberg N, Sommer C, European Federation of Neurological Societies. EFNS guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy. Eur J Neurol 2005;12:747–58. [PubMed: 16190912]
14. Leinninger GM, Edwards JL, Lipshaw MJ, Feldman EL. Mechanisms of disease: mitochondria as new therapeutic targets in diabetic neuropathy. Nat Clin Pract Neurol 2006;2:620–8. [PubMed: 17057749]

15. Leung AWC, Halestrap AP. Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. Biochim Biophys 2008;1777:946–52.

16. McEnery MW, Snowman AM, Trifiletti RR, Snyder SH. Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. Proc Natl Acad Sci (USA) 1992;89:3170–4. [PubMed: 1373486]

17. Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapere JJ, Lindemann P, Norenberg MD, Nutt D, Weizman A, Zhang MR, Gavish M. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006;27:402–9. [PubMed: 16822554]

18. Polomano R, Clark U, Mannes AJ, Bennett GJ. A painful peripheral neuropathy in rat produced by the chemotherapeutic drug, paclitaxel. Pain 2001;94:293–304. [PubMed: 11731066]

19. Rostovtseva TK, Bezrukov SM. VDAC regulation: role of cytosolic proteins and mitochondrial lipids. J Bioenerg Biomembr 2008;40:163–70. [PubMed: 18654841]

20. Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM, Sackett DL. Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. PNAS 2008;105:18746–51. [PubMed: 19033201]

21. Siau C, Bennett GJ. Dysregulation of neuronal calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy. Anesth Analg 2006;102:1485–90. [PubMed: 16632831]

22. Siau C, Xiao WH, Bennett GJ. Paclitaxel- and vincristine-evoked painful peripheral neuropathies: loss of epidermal innervation and activation of Langerhans cells. Exptl Neurol 2006;201:507–14. [PubMed: 16797537]

23. Varbiro G, Veres B, Gallyas F Jr. Sumegi B. Direct effect of Taxol on free radical formation and mitochondrial permeability transition. Free Radic Biol Med 2001;31:548–58. [PubMed: 11498288]

24. Wu G, Ringkamp M, Murinson BB, Pogatzki EM, Hartke TV, Weerahandi HM, Campbell JN, Griffin JW, Meyer RA. Degeneration of myelinated efferent fibers induces spontaneous activity in uninjured C-fiber afferents. J Neurosci 2002;22:7746–53. [PubMed: 12196598]

25. Xiao WH, Bennett GJ. Chronic inflammatory pain and persistent spontaneous discharge in A-fibers and C-fibers. Anesthesiology 2007;107:813–21. [PubMed: 18073557]

26. Xiao WH, Bennett GJ. Chemotherapy-evoked neuropathic pain: Abnormal spontaneous discharge in A-fiber and C-fiber primary afferent neurons and its suppression by acetyl-L-carnitine. Pain 2008a; 144:714–20.

27. Xiao WH, Bennett GJ. Low-frequency spontaneous discharge in primary afferent neurons innervating an inflamed muscle and its suppression by long-term nanogram plasma levels of lidocaine. Pain 2008b;135:262–70. [PubMed: 17659836]

28. Xiao WH, Naso L, Bennett GJ. Experimental studies of potential analgesics for the treatment of chemotherapy-evoked painful peripheral neuropathies. Pain Medicine 2008;9:505–17. [PubMed: 18777607]

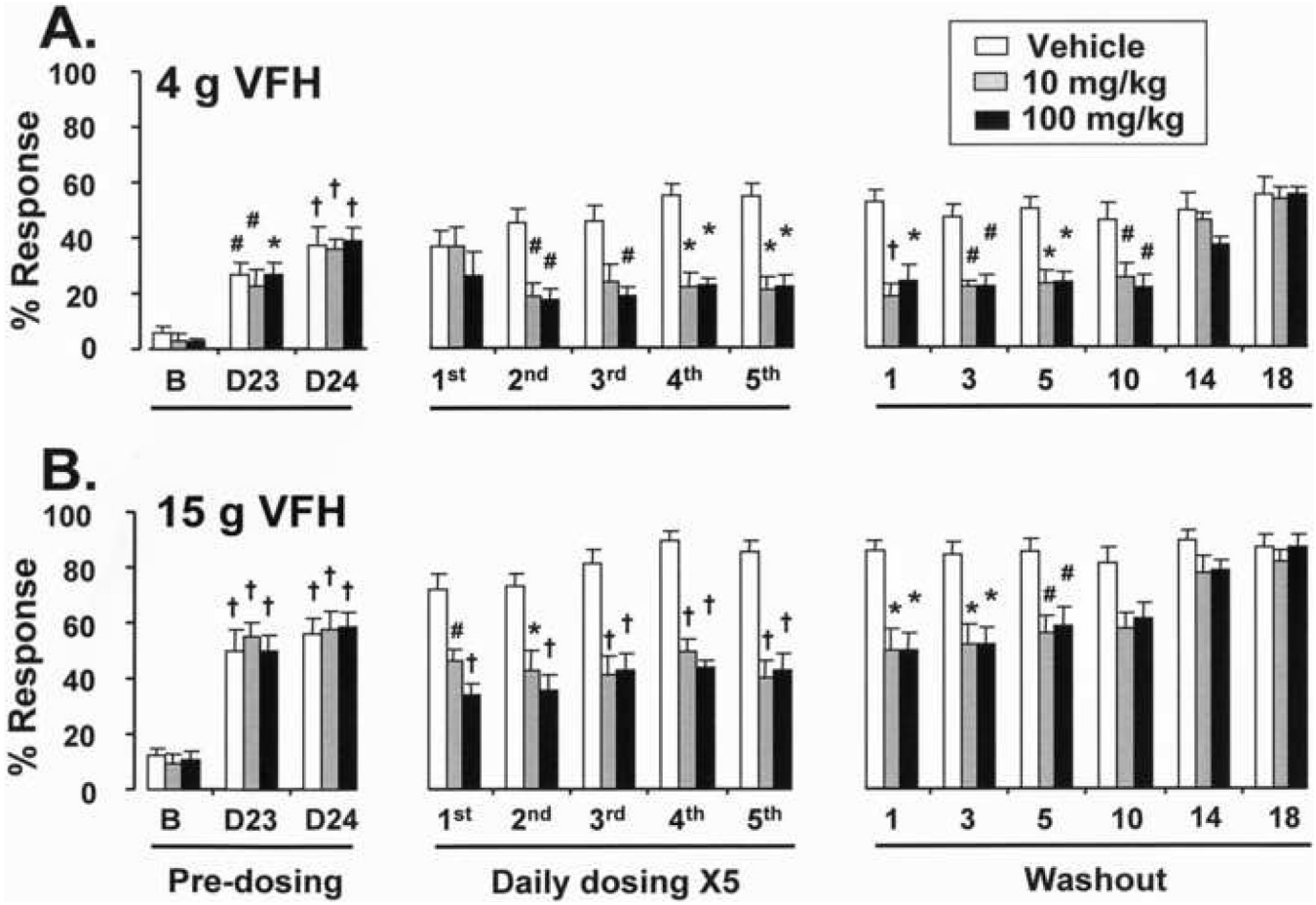

Fig. 1.

Treatment paradigm showing the effects of 5 daily doses of olesoxime on established paclitaxel-evoked neuropathic (A) mechano-allodynia (4 g VFH) and (B) mechano-hyperalgesia (15 g VFH). Pre-dosing: All three groups show the expected statistically significant mechano-allodynia and mechano-hyperalgesia on D23 and D24 after paclitaxel administration. Daily dosing: Effects of the 1^{st} – 5^{th} administrations of 10 and 100 mg/kg olesoxime. Washout days: Persistent anti-allodynia and anti-hyperalgesia following olesoxime administration. For each of the three phases of the study, the analysis was a repeated measures ANOVA followed by Bonferroni-corrected pair-wise comparisons. #,*, †: $p < 0.05, 0.01, 0.001$ *vs.* baseline (Pre-dosing) or vehicle control group (Daily dosing and Washout).

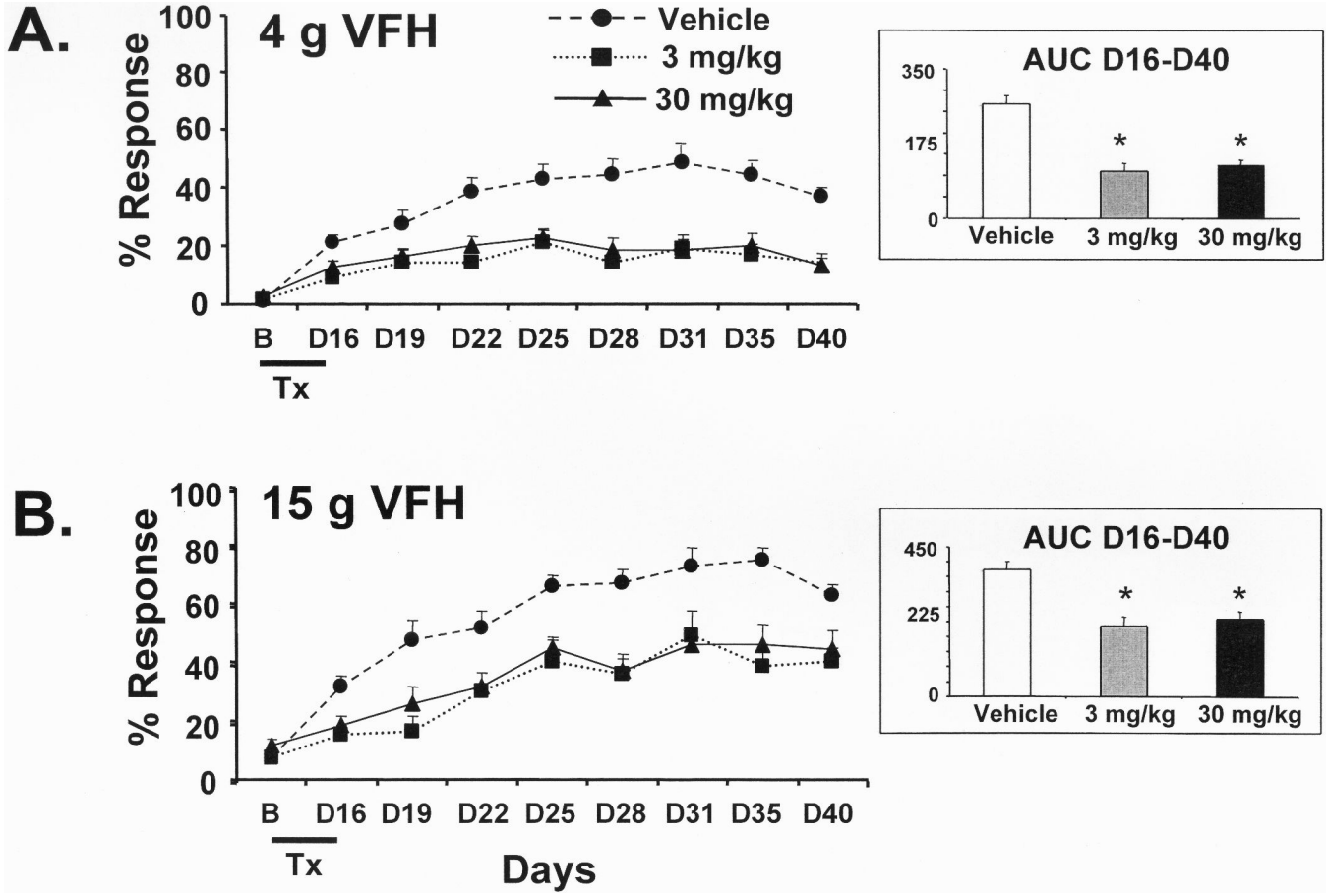

Fig. 2.

Preventive paradigm. Prophylactic dosing with 3 mg/kg and 30 mg/kg olesoxime significantly reduces the development of paclitaxel-evoked mechano-allodynia (A) and mechano-hyperalgesia (B). Tx: Olesoxime or vehicle were administered daily for 17 days, beginning the day before the first injection of paclitaxel and continuing for 9 days after the last paclitaxel injection. Insets: Area under the curve (AUC) analyses for D16-D40 (the ordinates show arbitrary units). One-way ANOVA followed by Dunnett's t-test; * $p < 0.01$ *vs.* vehicle.

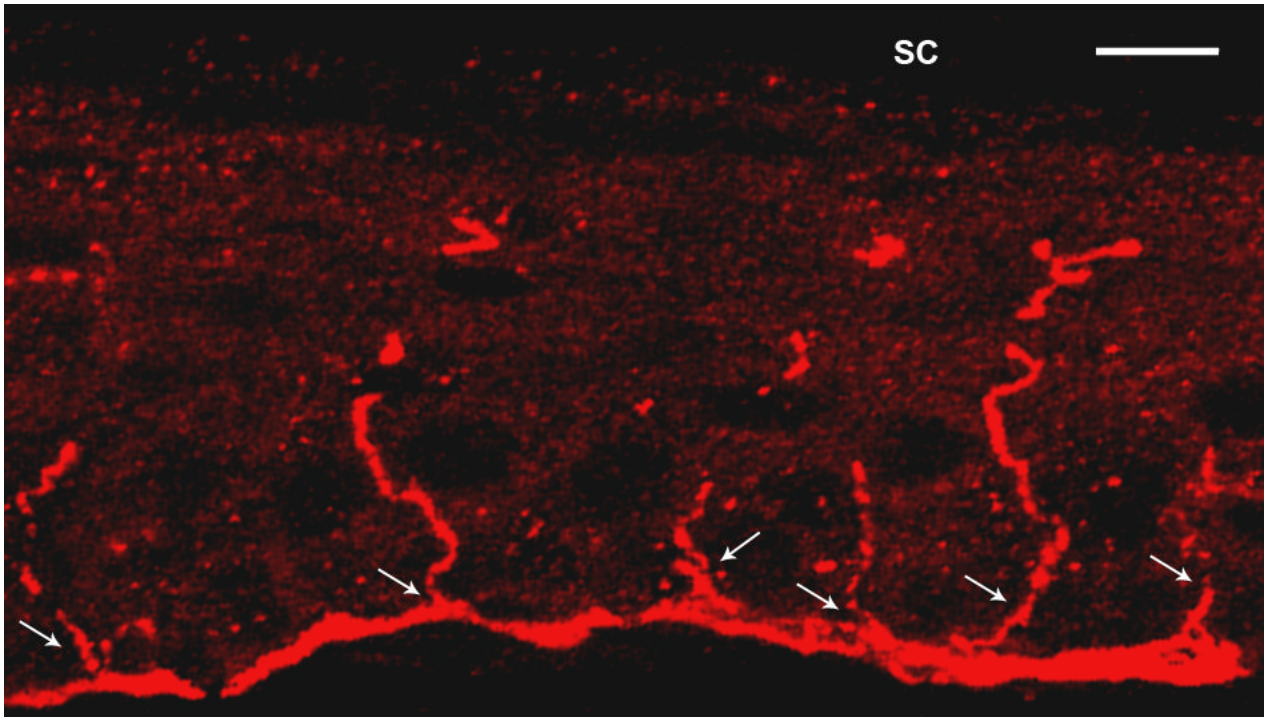

Fig. 3.
PGP9.5 staining in a 30 μm section of glabrous hind paw skin from a vehicle-injected rat. IENFs (arrows) arise from a subepidermal fiber plexus and cross into the epidermis where they ramify and issue terminal boutons throughout the vital layers of the epidermis. SC: stratum corneum. Scale bar: 20 μm.

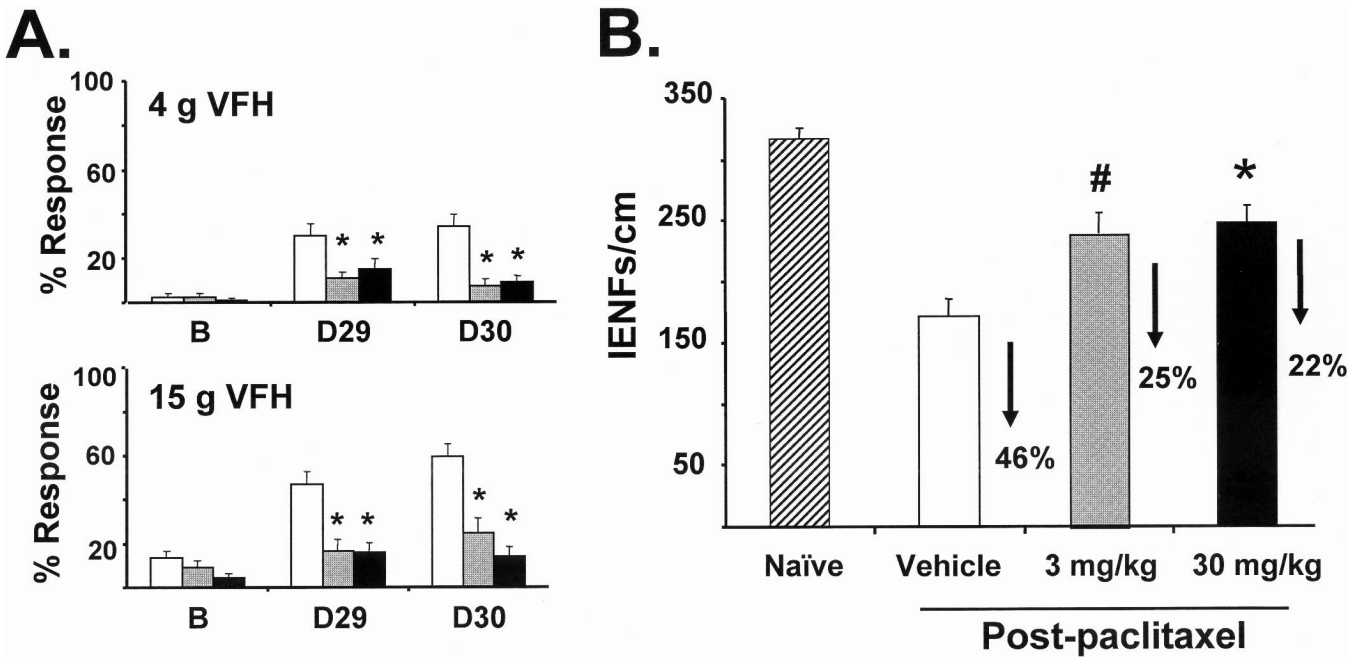

Fig. 4.

Effects of olesoxime on paclitaxel-evoked IENF degeneration. The dosing protocol was the same as for Fig. 2. (A) Behavioral assays on D29 and D30 confirmed the expected paclitaxel-evoked mechano-allodynia (top) and mechano-hyperalgesia (bottom) in the vehicle-treated groups and the expected analgesic effects in both olesoxime-treated groups (3 mg/kg and 30 mg/kg). * $p < 0.01$ *vs*. vehicle group; ANOVA followed by Bonferroni-corrected pair-wise comparisons . (B) IENF counts. Paclitaxel-treated animals treated with vehicle had the expected significant loss of IENFs ($p < 0.001$ *vs*. naïve controls). Paclitaxel-treated animals had significantly less IENF degeneration after treatment with both doses of olesoxime (#, *, $p < 0.05$, 0.01 *vs*. vehicle group; ANOVA followed by Bonferroni-corrected pair-wise comparisons). However, both olesoxime-treated groups still had significantly ($p < 0.05$) fewer IENFs than found in the naïve control group. Thus, olesoxime treatment produced a significant but partial protection against paclitaxel-evoked degeneration.

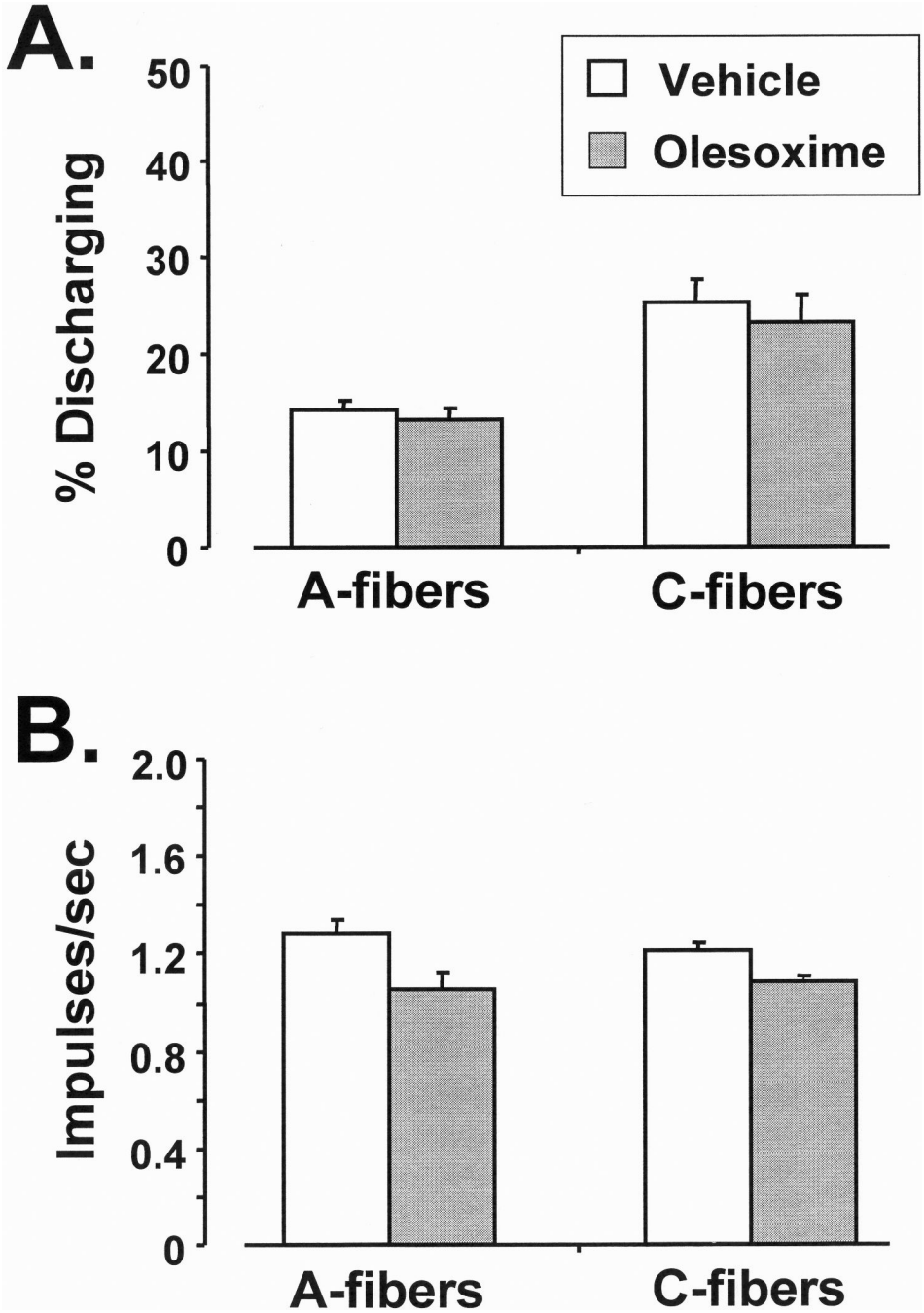

Fig. 5.

Effects on paclitaxel-evoked spontaneous discharge in A-fibers and C-fibers. (A) Paclitaxel-treated animals given vehicle had the expected significant increase ($p < 0.01$; χ^2 test) in the incidence of spontaneous discharge in both A-fibers and C-fibers relative to the incidence seen in naïve control animals (whose data are not shown). There was no significant difference in the incidence of spontaneous discharge in paclitaxel-treated animals receiving olesoxime compared to those receiving vehicle. (B) Spontaneous discharge frequencies for A-fibers and C-fibers. Olesoxime had no effect on the frequency of paclitaxel-evoked spontaneous discharge.