Targeting the Overproduction of Peroxynitrite for the Prevention and Reversal of Paclitaxel-Induced Neuropathic Pain

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Chemotherapy-induced peripheral neuropathy (CIPN) accompanied by chronic neuropathic pain is a major dose-limiting side effect of a large number of antitumoral agents including paclitaxel (Taxol). Thus, CIPN is one of most common causes of dose reduction and discontinuation of what is otherwise a life-saving therapy. Neuropathological changes in spinal cord are linked to CIPN, but the causative mediators and mechanisms remain poorly understood. We report that formation of peroxynitrite (PN) in response to activation of nitric oxide synthases and NADPH oxidase in spinal cord contributes to neuropathological changes through two mechanisms. The first involves modulation of neuroexcitatory and proinflammatory (TNF- α and IL-1 β) and anti-inflammatory (IL-10 and IL-4) cytokines in favor of the former. The second involves post-translational nitration and modification of glia-derived proteins known to be involved in glutamatergic neurotransmission (astrocyte-restricted glutamate transporters and glutamine synthetase). Targeting PN with PN decomposition catalysts (PNDs) not only blocked the development of paclitaxel-induced neuropathic pain without interfering with antitumor effects, but also reversed it once established. Herein, we describe our mechanistic study on the role(s) of PN and the prevention of neuropathic pain in rats using known PNDs (FeTMPyP⁵⁺ and MnTE-2-PyP⁵⁺). We also demonstrate the prevention of CIPN with our two new orally active PNDs, SRI6 and SRI110. The improved chemical design of SRI6 and SRI110 also affords selectivity for PN over other reactive oxygen species (such as superoxide). Our findings identify PN as a critical determinant of CIPN, while providing the rationale toward development of superoxide-sparing and “PN-targeted” therapeutics.

Introduction

Paclitaxel (Taxol) is a widely used chemotherapeutic indicated for treating breast, ovarian, and non-small cell lung carcinomas and Kaposi's sarcoma. Unfortunately, the dose-limiting side effect and leading cause of discontinuation of this highly efficacious antitumor drug is peripheral neuropathy accompanied by chronic neuropathic pain that resolves within weeks, months, or years after drug termination (Cata et al., 2006b; Farquhar-Smith, 2011). The clinical management of these patients becomes diffi-

cult as current pain drugs are marginally effective and display unacceptable side effects (Farquhar-Smith, 2011). Understanding the underlying causative mechanisms is of paramount significance to identifying novel ways to minimize this side effect and maximize antitumor effects.

Peroxynitrite (PN), the reaction product of superoxide (SO) and nitric oxide (NO) (Beckman et al., 1990), is a potent proinflammatory and pronociceptive species implicated in pain of several etiologies (for review, see Salvemini et al., 2011). In these settings, two glial pathways underlie PN's effects in spinal cord: increased TNF- α and IL-1 β contributing to hypersensitivity in dorsal horn neurons (for review, see DeLeo and Yeziarski, 2001; Watkins et al., 2001; Milligan and Watkins, 2009) and post-translational nitration of the glutamate transporter (GT), GLT-1, and glutamine synthetase (GS), key regulators of optimal synaptic glutamate homeostasis (Kugler, 1993). PN-mediated nitration of these proteins inactivates their biological function, thus enhancing glutamatergic neurotransmission (Trotti et al., 1996, 1999; Miñana et al., 1997; Görg et al., 2005). It is noteworthy that increased glial-derived cytokines (Ledeboer et al., 2006; Peters et al., 2007) and altered glutamatergic neurotransmission (Weng et

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al., 2005; Cata et al., 2006a; Zhang et al., 2012) are associated with paclitaxel-induced neuropathic pain; however, the mechanism(s) responsible remains largely unknown. Thus, it is possible that increased spinal PN following paclitaxel treatment triggers such neuropathological events. Our results reveal that overt spinal formation of PN following nitric oxidase synthase (NOS) and NADPH oxidase activation and manganese superoxide dismutase (MnSOD) inactivation contributes to neuropathic pain by increasing TNF- α and IL-1 β and engaging in post-translational nitration of GLT-1 and GS. Removing PN with well characterized peroxynitrite decomposition catalysts (PNDCs), FeTMPyP⁵⁺ and MnTE-2-PyP⁵⁺ (Salvemini et al., 1998; Batinic-Haberle et al., 2010), blocked these changes and neuropathic pain without interfering with antitumor effects and, importantly, reversed established pain.

Current PNDCs are nonselective with equal catalytic activities toward both PN and SO (Salvemini et al., 1998; Batinic-Haberle et al., 2010); making it difficult to decipher each species' contribution in pathophysiological settings. Moreover, the lack of oral bioavailability can restrict their chronic use (for review, see Salvemini et al., 2011). Evidence also indicates the importance of SO in learning and memory (Massaad and Klann, 2010), while PN has no known benefit. Therefore, targeting PN while sparing SO may provide a better strategy for designing molecules that dissect PN signaling pathways without compromising important physiological pathways. Our chemical design paradigm pairing PNDC selectivity with enhanced drug-like properties led to orally bioavailable and "SO-sparing" PNDCs, SRI110 (Rausaria et al., 2011a) and SRI6 (Rausaria et al., 2011b), which block and reverse neuropathic pain. Selective "PN-targeted" approaches may open new translational pathways for developing pain therapies and maximize chemotherapeutic doses.

Materials and Methods

Materials

Paclitaxel was purchased from Parenta Pharmaceuticals. FeTMPyP⁵⁺ was obtained from Cayman Chemical, whereas the MnTE-2-PyP⁵⁺ was provided by Dr. I. Batinic-Haberle (Duke University, Durham, NC). For cell culture: media (DMEM and McCoy's 5A) were purchased from Mediatech, FBS from Thermo Scientific Hyclone, and the penicillin/streptomycin from Invitrogen. For immunofluorescence, immunoprecipitation, and Western blot, the antibodies were from the following sources: anti-NT antibody was a kind gift from Dr. H. Ischiropoulos (The Children's Hospital of Philadelphia Research Institute, Philadelphia, PA); goat anti-rabbit rhodamine from Invitrogen; mouse monoclonal anti-glutamine synthetase from BD Biosciences; polyclonal rabbit anti-GLT-1 from Alpha Diagnostic; rabbit polyclonal anti-MnSOD from Millipore; mouse anti-GFAP from Santa Cruz Biotechnology; monoclonal mouse anti-OX-42 from Millipore; monoclonal mouse anti- β -actin from Sigma; and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies from Thermo Fisher Scientific. Fluoro-Gel II with DAPI was purchased from Electron Microscopy Sciences. The enhanced chemiluminescence reagents were from GE Healthcare Life Sciences (ECL) or Thermo Fisher Scientific. Rat brain lysate was purchased from Millipore. Unless otherwise stated, all other chemicals were purchased from Sigma.

Experimental animals

Male Sprague Dawley rats (200–220 g starting weight) from Harlan were housed 3–4 per cage in a controlled environment (12 h light/dark cycle) with food and water available *ad libitum*. All experiments were performed in accordance with the International Association for the Study of Pain and the National Institutes of Health guidelines on laboratory animal welfare and the recommendations by Saint Louis University Institutional Animal Care and Use Committee.

Chemotherapy-induced neuropathic pain

Paclitaxel or its vehicle (Cremophor EL and 95% dehydrated ethanol in 1:1 ratio) was injected intraperitoneally on four alternate days (2 mg/kg on days 0, 2, 4, and 6 with a final cumulative dose of 8 mg/kg) (Polomano et al., 2001). Experimental test substances or their vehicle were given either subcutaneously (s.c., 0.2 ml), orally by gavage (p.o., 0.2 ml) or by intrathecal injection (10 μ l followed by a 10 μ l flush with sterile physiological saline) in rats chronically implanted with intrathecal cannulas using the L5/L6 lumbar approach, as described previously by us and others (Størkson et al., 1996; Ledebor et al., 2007; Doyle et al., 2010; Muscoli et al., 2010) and commonly used for drug delivery (Schoeniger-Skinner et al., 2007; Doyle et al., 2010; Muscoli et al., 2010; Ramos et al., 2010). Test compounds were administered from day 0 (D0) (30 min before the first intraperitoneal injection of the chemotherapeutic agent) to D15 or were given once on D16.

Behavioral testing

The behavioral responses in this model last for months, thus modeling painful neuropathies in patients (Polomano et al., 2001). Behavioral testing was done with the experimenters blinded to treatment conditions. All animals were allowed to acclimate for ~15 min. If testing coincided with a day when rats received their drugs, behavioral testing was done always before the injection of the test substance.

Mechano-allodynia. Mechanical withdrawal thresholds were taken using calibrated von Frey filaments [Stoelting, ranging from 3.61 (0.407 g) to 5.46 (26 g) bending force] according to the "up-and-down" method (Dixon, 1980). The development of mechano-allodynia is evidenced by a significant ($p < 0.05$) reduction in mechanical mean absolute paw-withdrawal thresholds (g) at forces that failed to elicit withdrawal responses before the administration of the chemotherapeutic treatment (baseline).

Mechano-hyperalgesia. Mechanical withdrawal threshold was measured by the Randall and Sellitto paw pressure test (Randall and Sellitto, 1957) using an analgesimeter (Ugo Basile), which applies a linearly increasing mechanical force to the dorsum of the rat's hindpaw. The nociceptive threshold was defined as the force (g) at which the rat withdrew its paw (cutoff set at 250 g). Paclitaxel treatment results in bilateral allodynia and hyperalgesia (Polomano et al., 2001). Since the thresholds did not differ between left and right hindpaws at any time point in any group, values from both paws were averaged for further analysis and data presentation. None of the animals exhibited obvious signs of toxicities; i.e., they exhibited normal posture, grooming, locomotor behavior, hair coat was normal without signs of piloerection or porphyrin, and body weight gain was normal and comparable to vehicle-treated rats.

ED₅₀ determination

The effective dose providing 50% inhibition of allodynia or hyperalgesia was determined using a 4-parameter variable slope nonlinear regression analysis of the % Inhibition using GraphPad Prism v5.03. % Inhibition = $(PWT_{PNDC} - PWT_{paclitaxel}) / (PWT_{veh} - PWT_{paclitaxel}) \times 100$, where PWT = Paw Withdrawal Threshold, PNDC = peroxynitrite decomposition catalyst at a particular dose, and Veh = vehicle.

Immunofluorescence detection

After behavioral measurements, rats were anesthetized with ketamine/xylazine and intracardially perfused with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The spinal cord (L4–L6) was removed and fixation continued in the same fixative for 16 h at 4°C. After washing in PBS (pH 7.4), the tissue was infiltrated with 30% (w/v) sucrose in PBS at 4°C for 24 h, washed again in PBS, transferred to OCT, and snap frozen in liquid nitrogen. Transverse sections (10 μ m) were cut in a cryostat, collected on glass microscope slides, and stored at –20°C until used for immunofluorescent detection of nitrotyrosine (NT) as we reported previously (Wang et al., 2004; Muscoli et al., 2007, 2010). The sections were blocked, then labeled with a well characterized affinity-purified rabbit polyclonal anti-NT antibody (1:150) (Fries et al., 2003) in 1/10th blocking buffer for 3 h at room temperature (RT) or 16 h at 4°C. The sections were rinsed in PBS and target proteins detected with goat anti-rabbit rhodamine (1:400) in 1/10th blocking buffer for 1 h at RT in a humidified container. Following several PBS

rinsed, the sections were mounted in Fluoro-Gel II with DAPI, coverslipped, and photographed with an Olympus FV1000 confocal microscope at the same settings across all groups. Additional sections were treated with normal rabbit IgG at concentrations equivalent to those of the primary antibodies as controls and yielded only nonspecific background fluorescence. The immunohistochemical results shown in Figure 3 (see below) are representative examples of 3 or more separate immunostaining reactions in 3 different animals in which similar results were obtained. The micrographs were taken from the superficial layer of the dorsal horn of the spinal cord. Post-acquisition adjustments were applied equally between all groups. The results were analyzed by qualitative inspection of the staining patterns and fluorescence intensity in saved digital images.

Immunoprecipitation and immunoblotting

Cytosolic fractions and P2 membranes were obtained as previously described (Takagi et al., 2000; Wang et al., 2004; Muscoli et al., 2007) and stored immediately at -80°C . Immunoprecipitation and Western blot analyses were performed as described previously (Takagi et al., 2000; Wang et al., 2004; Muscoli et al., 2007). Proteins were resolved with 7.5% (GLT-1), 10% (glutamine synthetase), or 4–20% (MnSOD) SDS-PAGE before electrophoretic transfer. Membranes were blocked for 1 h at RT in 1% BSA in TBS-T (50 mM Tris-HCl, pH 7.4), 150 mM NaCl, 0.01% Tween-20 and 0.1% thimerosal) and then probed with mouse monoclonal anti-glutamine synthetase (1:2000), polyclonal rabbit anti-GLT-1 (1:1000), rabbit polyclonal anti-MnSOD (1:1000), mouse GFAP (1:1000), or mouse monoclonal OX-42 (1:1000). Membranes were washed with TBS/T and visualized with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT and enhanced chemiluminescence. Rat brain lysate containing our proteins of interest was used as positive control. The blots were stripped and probed with a murine monoclonal anti- β -actin antibody (1:2000). The relative density of the protein bands of interest were determined from film using ImageQuant 5.2 software (Molecular Dynamics) and normalized to β -actin bands.

Measurement of nitric oxide synthase activity

Spinal cords were homogenized in HEPES buffer (20 mM, pH 7.2, containing 320 mM sucrose, 1 mM DL-dithiothreitol, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin). The cytosolic fractions were collected by centrifuging at $100,000 \times g$ (L8–70 ultracentrifuge, Beckman) for 30 min at 4°C and stored at -70°C up to 10 d. Protein concentration in the cytosolic fractions was measured spectrophotometrically against a bovine serum albumin standard (Salter et al., 1991). NOS activity was evaluated by measuring the rate of conversion of L-[U - ^{14}C]arginine to citrulline, according to the method of Salter et al. (1991). Briefly, cytosolic fractions (100 μg of protein) were incubated for 10 min at 37°C in potassium phosphate buffer [50 mM, pH 7.2, containing (in mM): 60 L-valine, 120 μM NADPH, 1.2 L-citrulline, 1.2 MgCl_2 , and 0.24 CaCl_2 in the presence of test compounds (0.01–1 mM) or vehicle]. Samples were then incubated for 10 min at 37°C with L-[U - ^{14}C]arginine (150,000 dpm, specific activity 304 mCi/ μmol , NEN Life Science) and 20 μM L-arginine. The reaction was stopped by the addition of 1.0 ml of a mixture of H_2O /Dowex-50W 1:1 v/v (200–400, 8% cross-linked, H^+ -form). The Na^+ -form of Dowex-50W was prepared by washing the H^+ -form of resin four times with 1 M NaOH and then with bidistilled water until the pH was <7.5 . The sample/resin was centrifuged ($11,000 \times g$ for 3 min) and an aliquot of the supernatant was taken for scintillation counting (in 4 ml of Pico-Aqua; PerkinElmer). The activity of constitutive Ca^{2+} -dependent NOS was determined from the difference between the labeled citrulline produced by control samples and samples containing 1 mM EGTA; the activity of inducible Ca^{2+} -independent enzyme was determined from the difference between the labeled citrulline produced by samples containing 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM N^G -monomethyl-L-arginine. The activity of both isoforms was expressed as nmol/min/mg protein.

Measurement of NADPH oxidase activity

Spinal cord tissues (L4–L6) were homogenized in HEPES buffer (10 mM, pH 7.5, containing 250 mM sucrose, 1 mM EGTA, 25 mM potassium

chloride, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 2 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin) and centrifuged at $1000 \times g$ to obtain the nuclear-free supernatants. The NADPH cytochrome *c* reductase activity in these supernatants was measured using Cytochrome *c* Reductase (NADPH) Assay Kit following the manufacturer's instructions.

Measurement of mitochondrial MnSOD activities in spinal cord

Spinal cord tissues from lumbar region enlargement (L4–L6) were homogenized with 10 mM PBS, pH 7.4, sonicated on ice for 1 min (20 s, 3 times), and centrifuged at $1100 \times g$ for 10 min. The SOD activity in the supernatants was measured, as described previously (Wang et al., 2004), by the ability to competitively inhibit xanthine-xanthine oxidase-derived superoxide reduction of NBT to blue tetrazolium salt. Copper, zinc SOD (Cu,ZnSOD) activity was blocked in this assay by the addition of 2 mM NaCN after preincubation for 30 min. The rate of NTB reduction was monitored spectrophotometrically (PerkinElmer Lambda 5 Spectrophotometer) at 560 nm. The amount of protein required to inhibit the rate of NTB reduction by 50% was defined as one unit of enzyme activity. Enzymatic activity was expressed in units per milligram of protein (Wang et al., 2004).

Cytokine ELISA

Spinal cord levels of TNF- α , IL-1 β , IL-10, and IL-4 were measured using commercially available ELISA kits (R&D Systems) according to the manufacturer's protocol.

Antitumor activity of paclitaxel

The effects of MnTE-2-PyP $^{5+}$ on antitumor activity of paclitaxel on well characterized human breast cancer cells (SKBR3) (Swift et al., 2010; Itamochi et al., 2011) and human ovarian cancer cells (SKOV3) (Lacroix and Leclercq, 2004; Swift et al., 2010; Wu et al., 2010) was assessed using an MTT assay adapted from a previously described assay (Dahan et al., 2009; Kriedt et al., 2010). Cells were cultured and assayed at 37°C , 95% humidity, and 5% CO_2 in DMEM (SKBR3) or McCoy's 5A (SKOV3) medium, each supplemented with 10% FBS and penicillin/streptomycin. Cells (3.13×10^4 cells/ cm^2) were plated in 12-well plates (Griener Bio-One) in complete media and incubated overnight. This plating regimen yielded 60% confluent cultures for testing. The cells were equilibrated in fresh media (5 h) and treated with 60 μM MnTE-2-PyP $^{5+}$ or its vehicle (saline), then with paclitaxel (1, 10, 100, 1000 nM) or its vehicle (1% of 522 mg of Cremophor in 43.2% ethanol). The cultures were incubated for 48 h and the level of cell survival was assessed by treating the cells with 500 $\mu\text{g}/\text{ml}$ MTT for 75 min, 37°C . The medium was removed and the tetrazolium crystals were dissolved in isopropanol. The $A_{570\text{ nm}}$ was measured using a Genesis5 spectrophotometer (Thermo Fisher Scientific). % Survivability = ($A_{570\text{ nm}}$ of the test well)/($A_{570\text{ nm}}$ veh) $\times$ 100. The concentration providing 50% lethality (LC_{50}) for paclitaxel with or without MnTE-2-PyP $^{5+}$ was calculated using a three-parameter nonlinear analysis of % Survivability where the top and bottom plateaus were constrained using GraphPad Prism v5.03.

Superoxide dismutase activity of PNDCs

The SOD activity of PNDCs was determined by their competitive inhibition of luminal oxidation by xanthine/xanthine oxidase-derived superoxide (Bensinger and Johnson, 1981; Kimura and Nakano, 1988; Decraene et al., 2004). Serially diluted compounds were tested in duplicate by adding 50 μl of compound/vehicle [$4 \times$ in TE buffer: 50 mM Tris-HCl, pH 7.8, and 1 mM EDTA] and 50 μl of xanthine oxidase from bovine milk, Grade III (0.4 U/ml in TE) to a 96-well white-walled microplate. A twofold serially diluted Cu,ZnSOD standard curve (0.25–260 U/ml) was similarly prepared. In a Glomax Multidetection System (Model 9301, Promega) primed with xanthine (200 μM in TE buffer) and luminol (600 μM in TE), and xanthine (50 μl) was automatically injected and read for 1 s following 1 s delay (background signal); then luminol (50 μl) was injected and read for 1 s following 1 s delay (luminol signal). The final concentration of each component was compound/vehicle (1 $\times$), xanthine oxidase (0.1 U/ml), luminol (150 μM), and xanthine (50 μM). The luminol signal was corrected for background signal and the SOD-like activity/ml of each compound was determined from the log-log linear regression analysis of Cu,ZnSOD standard curve. The SOD activity per

milligram (U/mg) of compound was measured as the slope of the U/ml of serially diluted compound versus the concentration of compound (mg/ml). All linear analyses were performed using GraphPad Prism v5.03 with significance set at $p < 0.05$.

Peroxynitrite decomposition via inhibition of aryl boronate oxidation assay

Stock solutions of 4-acetylphenylboronic acid and the catalyst were prepared in DMSO (in the 5–50 mM range). Peroxynitrite in 0.1N NaOH solution was prepared by the method of Pryor (Uppu et al., 1996) and frozen at -80°C until needed. Small aliquots of the PN solution were thawed and kept on ice, and the concentration was measured by UV spectroscopy just before use. Peroxynitrite concentrations ranged from 58 to 77 mM for these studies. In a typical procedure, 9.5×10^{-7} mol of 4-acetylphenylboronic acid (24.0 μl of stock) were dispensed into a small vial equipped with a magnetic stir bar. Phosphate buffer (2.00 ml, 100 mM, pH = 7.2) containing 0.7% SDS and 100 μM diethylenetriamine pentaacetic acid was added followed by 9.5×10^{-7} mol of the catalyst (aliquot from DMSO stock). To this rapidly stirred mixture, PN (9.5×10^{-7} mol) was rapidly injected. The mixture was stirred for 1 min and analyzed by liquid chromatography [Waters Alliance-MS3100 system; 15% acetonitrile/ H_2O to 95% acetonitrile (0.05% trifluoroacetic acid) over 10 min; Agilent Eclipse XD8-C18 column, 5 μM , 4.6×150 mm, UV detection 280 nm for 4-hydroxyacetophenone oxidation product]. Replicate reactions ($n = 5$) were run and compared with controls (also $n = 5$), which contained everything except the catalyst (amounts of DMSO that were equivalent to those from aliquoted catalyst solutions were added to the controls to compensate for the very small effect of DMSO). The peak areas for phenol oxidation products were compared for catalyst versus control runs to determine percentage inhibition.

Calculations:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OX}_{\text{cat}}}{\text{OX}_{\text{control}}}\right) \times 100 \left(1 - \frac{\text{OX}_{\text{cat}}}{\text{OX}_{\text{control}}}\right) \times 100$$

$$\% \text{ RSD}^2 = (\% \text{RSD OX}_{\text{cat}})^2 + (\% \text{RSD OX}_{\text{control}})^2 + (\% \text{RSD OX}_{\text{cat}})^2 + (\% \text{RSD OX}_{\text{control}})^2$$

$$\Delta\%I = \sqrt{\left(\frac{\Delta\text{OX}_{\text{cat}}}{\text{OX}_{\text{cat}}}\right)^2 \times \left(\frac{\Delta\text{OX}_{\text{control}}}{\text{OX}_{\text{control}}}\right)^2} \times \%I \sqrt{\left(\frac{\Delta\text{OX}_{\text{cat}}}{\text{OX}_{\text{cat}}}\right)^2 \times \left(\frac{\Delta\text{OX}_{\text{control}}}{\text{OX}_{\text{control}}}\right)^2} \times \%I$$

Estimated k for oxidation of Mn(III) to Mn(V)=O:

$$k_{\text{est}} = \left(\frac{\%I}{100 - \%I}\right) \times 1.6 \times 10^6 k_{\text{est}} = \left(\frac{\%I}{100 - \%I}\right) \times 1.6 \times 10^6 \text{ (rate of oxidation of boronic acid by peroxynitrite)}$$

$$\% \text{ RSD}^2 = (\% \text{RSD OX})^2 + (\% \text{RSD OX}_{\text{reduced}})^2 = (\% \text{RSD OX})^2 + (\% \text{RSD OX}_{\text{reduced}})^2$$

$$\Delta k_{\text{est}} = \sqrt{\left(\frac{\Delta\%I}{\%I}\right)^2 + \left(\frac{\Delta\%I}{100 - \%I}\right)^2} \times k_{\text{est}} \Delta k_{\text{est}} = \sqrt{\left(\frac{\Delta\%I}{\%I}\right)^2 + \left(\frac{\Delta\%I}{100 - \%I}\right)^2} \times k_{\text{est}}$$

Statistical analysis

Data are expressed as mean $\pm$ SD and analyzed with two-tailed, two-way repeated measures ANOVA with Bonferroni test or one-way ANOVA with Dunnett's test against paclitaxel group, where noted. Differences

Figure 1. Targeting PN blocks neuropathic pain. **A–F**, Compared with vehicle (○), administration of paclitaxel (●) led to a time-dependent development of mechano-allodynia and mechano-hyperalgesia that was attenuated by daily subcutaneous injections (D0–D15) of L-NAME (100 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$; ■, **A, B**), L-NIL (30 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$; ▼, **A, B**), apocynin (100 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$; ▲, **A, B**), or FeTMPyP⁵⁺ (**C, D**) and MnTE-2-PyP⁵⁺ (**E, F**) at 1, ■, ▼; or 10 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, ▲, △. These drugs did not affect withdrawal thresholds in vehicle groups (**A, B**: L-NAME, □; L-NIL, ▽; and apocynin, △; **C, D**: FeTMPyP⁵⁺, □; **E, F**: MnTE-2-PyP⁵⁺, □). Results are expressed as mean $\pm$ SD for 6 rats and analyzed by two-tailed, two-way repeated-measures ANOVA with Bonferroni comparisons. * $p < 0.001$ for paclitaxel versus vehicle and $^{\dagger}p < 0.001$ for paclitaxel versus paclitaxel + experimental drugs.

Figure 2. Neuropathic pain does not develop upon PNDC discontinuation. When compared with vehicle (○), administration of paclitaxel (●) led to a time-dependent development of mechano-allodynia (**A**) and mechano-hyperalgesia (**B**). These events were attenuated by daily subcutaneous injections (D0–D15) of FeTMPyP⁵⁺ (■) and MnTE-2-PyP⁵⁺ (▲) at 10 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. Moreover, mechano-allodynia and mechano-hyperalgesia did not develop over an additional 10 d through D25 following discontinuation of FeTMPyP⁵⁺ (■) and MnTE-2-PyP⁵⁺ (▲). Results are expressed as mean $\pm$ SD for 6 rats and analyzed by two-tailed, two-way repeated-measures ANOVA with Bonferroni comparisons. * $p < 0.001$ for paclitaxel versus vehicle and $^{\dagger}p < 0.01$ or $^{\ddagger}p < 0.001$ for paclitaxel versus paclitaxel + experimental drugs.

between dose–response curves for antitumor activity were analyzed by 3-parameter nonlinear analysis with constrained top and bottom plateaus and Hill Plot slopes. Dose–response curves for SOD activity were analyzed by linear regression analysis. Significance was accepted at $p <$

Figure 3. Neuropathic pain increases nitric oxide synthase and NADPH oxidase activation and PN formation in the spinal dorsal horn. **A–E**, Compared with vehicle, paclitaxel increased the activity of constitutive/calcium-dependent NOS (black bars) and inducible/calcium-independent NOS (gray bars) (**A**), and NADPH (**B**). L-NAME (100 mg · kg^{−1} · d^{−1}) blocked constitutive/calcium-dependent NOS (**A**, black bars) activity, whereas L-NAME and L-NIL (30 mg · kg^{−1} · d^{−1}) inhibited inducible/calcium-independent NOS (**A**, gray bars) activity. Paclitaxel also increased NADPH oxidase activity, which was attenuated with apocynin (100 mg · kg^{−1} · d^{−1}, **B**). As indicated by the formation of NT (red) in fixed frozen spinal cord sections (**C–E**), PN formation was low in dorsal horn from vehicle-treated animals (**C**) but substantially increased with paclitaxel treatment (**D**). This increase was attenuated by MnTE-2-PyP⁵⁺ (10 mg · kg^{−1} · d^{−1}, **E**). **C–E** are single-plane confocal images. The inset in **D** is a projected image of an 18 plane Z-stack to illustrate the morphology of a NT-positive cell in the volume of a 10 μm dorsal horn section. Negative controls using normal rabbit serum for NT exhibited only low levels of background fluorescence. Micrographs are taken from superficial layers of dorsal horn of the spinal cord (lumbar enlargement) and are representative of at least 3 images from 3 different animals performed on different days. Enzymatic activities are mean ± SD for 6 rats and analyzed by two-tailed, one-way ANOVA with Dunnett's comparisons to paclitaxel group. **p* < 0.001 for paclitaxel versus Veh; †*p* < 0.01 or ††*p* < 0.001 for paclitaxel versus paclitaxel + experimental drugs.

0.05. All statistical analysis was performed using GraphPad Prism (Release 5.03).

Results

Targeting PN blocks the development of paclitaxel-induced neuropathic pain

Confirming previous reports (Polomano et al., 2001; Flatters and Bennett, 2006; Ledebor et al., 2007), our experiments revealed that neuropathic pain (mechano-allodynia and mechano-hyperalgesia) was significant by D11–D12 (onset, Figs. 1, 2), peaked by D16 (Figs. 1, 2) and plateaued throughout our observation period (D25; Fig. 2). Although chemotherapy dosing is completed within a few days, we continued dosing until the time when the pain occurs. The delay to symptom onset (also noted in patients) introduces uncertainty about the time of onset of the relevant pathological process. Continuing treatment until D15 is thus prudent. When compared with the vehicle group, administration of paclitaxel led to the development of mechano-allodynia and mechano-hyperalgesia (Fig. 1) that was attenuated by daily administration of a nonselective NOS inhibitor, L-NAME [100 mg · kg^{−1} · d^{−1}, s.c., *n* = 6; Fig. 1A,B], a more iNOS-selective inhibitor *N*⁶-(1-iminoethyl)-L-lysine (L-NIL; 30 mg · kg^{−1} · d^{−1}, s.c., *n* = 6; Fig. 1A,B) (Moncada et al., 1991), or by a well characterized NADPH oxidase inhibitor (apocynin, 100 mg · kg^{−1} · d^{−1}, s.c., *n* = 6; Fig. 1A,B) (Simons et al., 1990). When given alone, L-NAME, L-NIL or apocynin did not affect baseline withdrawal thresholds (Fig. 1A,B). Since NO is known to

react with SO at a near diffusion-limited rate to form PN (Beckman et al., 1990), these results indirectly suggest that PN generated from these species is the common denominator leading to neuropathic pain. The role of PN in CIPN was established using the well characterized iron and manganese porphyrin-based PNDs, FeTMPyP⁵⁺ and MnTE-2-PyP⁵⁺ (Salvemini et al., 1998; Batinic-Haberle et al., 2010). Thus, subcutaneous injections of FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ attenuated the development of neuropathic pain in a dose-dependent manner (1–10 mg · kg^{−1} · d^{−1}, *n* = 6; Fig. 1C,D; E,F, respectively). The ED₅₀ (effective dose providing 50% effect) of FeTMPyP⁵⁺, as calculated on D16 for mechano-allodynia and mechano-hyperalgesia, was 2.2 and 2.8 mg · kg^{−1} · d^{−1} and that for MnTE-2-PyP⁵⁺ was 3.1 and 2.7 mg · kg^{−1} · d^{−1}. When given alone, and at the highest dose tested (10 mg · kg^{−1} · d^{−1}, *n* = 6), FeTMPyP⁵⁺ (Fig. 1C,D) or MnTE-2-PyP⁵⁺ (Fig. 1E,F) did not affect baseline withdrawal thresholds. To determine whether CIPN, once it is prevented from developing, emerges after discontinuation of the drug, rats were treated for a period of 15 d with FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ (10 mg · kg^{−1} · d^{−1}, *n* = 6) and mechano-allodynia and hyperalgesia subsequently evaluated for an additional 10 d through D25. As can be seen in Figure 2, when treatment with FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ was discontinued on D15 neuropathic pain did not emerge.

Paclitaxel-induced neuropathic pain is associated with activation of nitric oxide synthases and NADPH oxidase in spinal cord

When compared with vehicle (*n* = 6), the development of neuropathic pain at the time of peak (D16) was associated with increased activation of the calcium-dependent/constitutive and -independent/inducible NOS activity (Fig. 3A). L-NAME (100 mg · kg^{−1} · d^{−1}, *n* = 6), but not L-NIL (30 mg · kg^{−1} · d^{−1}, *n* = 6), blocked constitutive NOS, whereas both inhibited inducible NOS (Fig. 3A), as expected. Significantly increased activation of NADPH oxidase and SO production in spinal cord was also observed and this was blocked by apocynin (100 mg · kg^{−1} · d^{−1}, *n* = 5, Fig. 3B).

Peroxynitrite formation and post-translational tyrosine nitration of mitochondrial superoxide dismutase, glutamate transporters and glutamine synthetase

Activation of NOS and NADPH oxidase are important in the biosynthesis of PN as they provide its precursors, NO and SO (Beckman et al., 1990). The highly reactive nature of PN, its formation, and its decomposition prevents direct measurements of PN *in vivo* and therefore, the detection of the formation of 3-nitrotyrosine (NT), is widely used for as a “footprint marker” to verify its presence provided that its expression is prevented by PNDs (Szabó et al., 2007). As can be seen in Figure 3, when compared with the vehicle group (Fig. 3C), the formation of NT increased in the dorsal horn of paclitaxel-treated animals

(Fig. 3D) and this was attenuated in rats treated with MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹, *n* = 3, s.c., Fig. 3E). Once formed, PN nitrates and inactivates mitochondrial MnSOD, the key enzyme keeping SO and thus PN in check (McCord and Fridovich, 1969). PN-mediated nitration and inactivation of MnSOD is an additional significant source in maintaining elevated levels of PN at pathophysiological sites (Macmillan-Crow and Cruthirds, 2001). The occurrence of this nitrated MnSOD in the spinal cord has been also linked to development of central sensitization in several experimental models of pain (for review, see Salvemini et al., 2011). We now show that this process is operative in paclitaxel-induced neuropathic pain as shown in Figure 4. Significant nitration of MnSOD was observed in spinal cord of paclitaxel rats (Fig. 4A) and this was associated with a significant loss in its ability to enzymatically dismutate SO (Fig. 4B). MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹, *n* = 6, s.c.) prevented MnSOD nitration (Fig. 4A) and its inactivation (Fig. 4B). The enzymatic activity of cytosolic Cu,ZnSOD remained unaffected (Table 1). Furthermore, we found that two proteins normally restricted to astrocytes and responsible for regulating glutamatergic signaling were nitrated in spinal cords from paclitaxel-treated rats, namely GLT-1 (Fig. 4C, *n* = 6) and GS (Fig. 4D, *n* = 6). Such post-translational nitration and modification was blocked in rats treated with MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹, *n* = 6; Fig. 4C,D).

The development of neuropathic pain is associated with increased PN-sensitive glial expression of GFAP and OX-42 in spinal cord

When compared with the vehicle group, the development of neuropathic pain was associated with enhanced expression of the astrocyte-associated GFAP and microglia associated OX-42 expression (Fig. 5A,B, *n* = 4). MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹) significantly reduced GFAP and OX-42 protein expression (Fig. 5A,B, *n* = 4).

PN contributes to the development of neuropathic pain through modulation of pro- and anti-inflammatory cytokines in spinal cord

When compared with the vehicle group, the development of neuropathic pain was associated with enhanced formation of TNF- α and IL-1 β (Fig. 5C,D, *n* = 4) and a small, but significant, increase in IL-10 and IL-4 (Fig. 5E,F, *n* = 5). MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹) significantly reduced TNF- α and IL-1 β (Fig. 5C,D, *n* = 4), whereas it significantly increased the levels of IL-10 and IL-4 (Fig. 5E,F, *n* = 5).

Intrathecal delivery of MnTE-2-PyP⁵⁺ blocks the development of neuropathic pain

Intrathecal delivery of FeTMPyP⁵⁺ (0.1–1 nmol/d, *n* = 6; Fig. 6A,B) or MnTE-2-PyP⁵⁺ (0.1–1 nmol/d, *n* = 6; Fig. 6C,D) at-

Figure 4. Attenuation of post-translational nitration of MnSOD, GLT-1, and GS by MnTE-2-PyP⁵⁺. **A–D**, Compared with vehicle, paclitaxel led to post-translational nitration (**A**) and enzymatic inactivation of mitochondrial MnSOD (**B**), events blocked by daily subcutaneous injections (D0–D15) of MnTE-2-PyP⁵⁺ (1–10 mg · kg⁻¹ · d⁻¹). Moreover, compared with vehicle, paclitaxel led to significant nitration of the glutamate transporter GLT-1 (**C**) and GS (**D**) in spinal cord tissues, events attenuated in rats treated with MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹; **C**, **D**). Results are expressed as mean $\pm$ SD for 6 rats and analyzed by two-tailed, one-way ANOVA with Dunnett's comparisons to paclitaxel group. **p* < 0.05 or ***p* < 0.001 for paclitaxel versus Veh; †*p* < 0.01 or ††*p* < 0.001 for paclitaxel versus paclitaxel + MnTE-2-PyP⁵⁺.

Table 1. Cu,ZnSOD activity

	Cu,ZnSOD Activity (U/ml)
Veh	209 $\pm$ 7.4
P	206 $\pm$ 5.5
P + MnTE-2-PyP ⁵⁺	
1 mg · kg ⁻¹ · d ⁻¹	208 $\pm$ 8.7
3 mg · kg ⁻¹ · d ⁻¹	207 $\pm$ 7.0
10 mg · kg ⁻¹ · d ⁻¹	200 $\pm$ 7.1
Veh + MnTE-2-PyP ⁵⁺ (10 mg · kg ⁻¹ · d ⁻¹)	210 $\pm$ 0.4

Mean $\pm$ SD for *n* = 6 rats.

tenuated the development of mechano-allodynia and mechano-hyperalgesia in a dose-dependent fashion. In addition, injections of MnTE-2-PyP⁵⁺ (1 nmol/d, *n* = 6) blocked the increased formation of TNF- α and IL-1 β (Fig. 6E,F, *n* = 6), but increased the formation of IL-10 and IL-4 (Fig. 6G,H, *n* = 6).

Systemic or intrathecal delivery of FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ reverse paclitaxel-induced neuropathic pain

To test whether the removal of PN reverses neuropathic pain once established, FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ (or their vehicle, saline) was given intraperitoneally (10 mg/kg, *n* = 6, Fig. 7A,B) or intrathecally (1 nmol/kg, *n* = 6, Fig. 7C,D) on D16. FeTMPyP⁵⁺ and MnTE-2-PyP⁵⁺ rapidly (within 30 min) and completely reversed mechano-allodynia and hyperalgesia which lasted at least for 5 h (Fig. 7); these effects were lost at the 24 h time point (data not shown). Doses of FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ were chosen from pilot dose-ranging experiments and used at a dose found to cause near-to-maximal reversal.

Figure 5. MnTE-2-PyP⁵⁺ attenuates GFAP, OX-42, and pro-inflammatory cytokine expression and favors anti-inflammatory cytokine production. **A–D**, Compared with vehicle (Veh), paclitaxel (P) increased GFAP (**A**), OX-42 (**B**), TNF-α (**C**), and IL-1β (**D**) expression ($n = 4$), which was significantly attenuated by MnTE-2-PyP⁵⁺ ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, i.p.). **E, F**, Compared with Veh, paclitaxel (P) increased anti-inflammatory IL-10 and IL-4 (**E, F**, $n = 5$) and was substantially enhanced by MnTE-2-PyP⁵⁺ ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, i.p.). Results are expressed as mean $\pm$ SD for (n) rats and analyzed by two-tailed, one-way ANOVA with Dunnett's comparisons to paclitaxel group. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ for paclitaxel versus Veh; † $p < 0.001$ for paclitaxel versus paclitaxel + MnTE-2-PyP⁵⁺.

MnTE-2-PyP⁵⁺ does not interfere with the antitumor effects of paclitaxel

MnTE-2-PyP⁵⁺ was chosen as a prototype agent since it catalytically decomposes both SO and PN (with preference toward PN in biological compartments in which both SO and NO are generated) and importantly because we had pharmacokinetic information to guide appropriate dose selection (Spasojević et al., 2008). In rats following a systemic injection of MnTE-2-PyP⁵⁺ at a dose providing near-to-maximal inhibition of neuropathic pain (10 mg/kg), plasma levels at C_{max} were $17 \mu\text{M}$ (Spasojević et al., 2008). Using a concentration 3 times higher than C_{max} ($60 \mu\text{M}$), the effect of MnTE-2-PyP⁵⁺ on the antitumor effects of paclitaxel was measured by the MTT assay (Shah et al., 2009; Kriedt et al., 2010) in the well characterized human breast (SKBR3) (Itamochi et al., 2011) and ovarian (SKOV3) (Lacroix and Leclercq, 2004; Wu et al., 2010) cancer cells. The antitumor activity of paclitaxel in SKBR3 ($\text{LC}_{50} = 9.6 \text{ nM}$) and SKOV3 ($\text{LC}_{50} = 14.0 \text{ nM}$) cells was not hindered, but if anything, enhanced by MnTE-2-PyP⁵⁺ ($\text{LC}_{50} = 4.2 \text{ nM}$; $p < 0.05$ and 5.7 nM $p < 0.001$ in SKBR3 and SKOV3 cells, respectively; $n = 4$).

SRI6 and SRI110 are novel orally active “SO-sparing” PNDCs

To test feasibility of engineering “SO-sparing PNDCs” with properties inherent of oral bioavailability, we selected a chemical design paradigm for which PN decomposition selectivity (over SO) and drug properties run in parallel. Our efforts to identify such a catalyst led to the discovery of two PNDCs of distinct classes: our previously reported SRI110 (Rausaria et al., 2011a)

and SRI6 (Rausaria et al., 2011b) (Fig. 8A). SRI110 is a neutral Mn(III) complex from the novel bis-hydroxyphenyldipyromethene class and SRI6 is an electron rich Mn(III) porphyrin system. To measure (under varying conditions) the PN decomposition activity of complexes designed to be membrane soluble, we developed an *in vitro* assay based upon the inhibition of aryl boronate oxidation by PN (Sikora et al., 2009). As can be seen from Figure 8B, stoichiometric SRI6 shows $30.8 \pm 2.3\%$ inhibition of the oxidation of 4-acetylphenylboronic acid by PN at 25°C . This corresponds to an apparent estimated second order rate constant of $7.1 \pm 0.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for the oxidation of SRI6 to the corresponding oxoMn(IV) species with the concomitant 1-electron reduction of PN. Ebselen (Sies and Masumoto, 1997) and Mn-4-TMPyP⁵⁺ (Ferrer-Sueta and Radi, 2009) were used as controls since their second order rate constants with PN are well known (Fig. 8B). The estimated second order rate constants for each of these compounds as determined by this assay match well with these published values (Fig. 8B). The rapid *in vivo* reduction of the resultant oxoMn(IV) form of SRI6 by endogenous antioxidants (ascorbate, glutathione, etc.) should complete a reductase-type catalytic cycle (Ferrer-Sueta and Radi, 2009). Similar values were found with the 2-electron PNDC, SRI110 (this complex is oxidized to the oxoMn(V) species with concomitant 2-electron reduction of PN). The carefully chosen chemical design features of SRI6 or SRI110 (Rausaria et al., 2011a) give it membrane solubility and reduce its activity toward SO by adjustment of the redox potential of the metal center. Importantly and as previously shown with SRI110, SRI6 behaves as a “SO-sparing

Figure 6. Intrathecal (i.th.) delivery of SODm/PNDCs blocks the development of neuropathic pain and modulates spinal cytokine production. **A–D**, Compared with vehicle (Veh, ○), paclitaxel (P, ●) led to a time-dependent development of mechano-allodynia (**A**, **C**) and mechano-hyperalgesia (**B**, **D**) that was attenuated by daily i.th. delivery of FeTMPyP⁵⁺ (**A**, **B**) or MnTE-2-PyP⁵⁺ (**C**, **D**) at 0.1, 0.3, 0.5, and 1 nmol/d, i.th. These drugs did not affect withdrawal thresholds in vehicle groups (□, **A–D**). **E–H**, Compared with vehicle group, paclitaxel increased TNF-α and IL-1β (**E**, **F**) and this was inhibited by MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹, i.th.). **G**, **H**, Paclitaxel led to a modest increase in IL-10 and IL-4 (**G**, **H**) production compared with vehicle; but was substantially enhanced with daily i.th. delivery of MnTE-2-PyP⁵⁺ (10 mg · kg⁻¹ · d⁻¹). Results are expressed as mean ± SD for 6 rats. Behavioral data were analyzed by two-tailed, two-way repeated-measures ANOVA with Bonferroni comparisons. Data for cytokine production were analyzed by two-tailed, one-way ANOVA with Bonferroni comparisons to paclitaxel group. **p* < 0.01 or ***p* < 0.001 for paclitaxel versus vehicle; †*p* < 0.01 or ‡*p* < 0.001 for paclitaxel versus paclitaxel + MnTE-2-PyP⁵⁺.

PNDC". Indeed, the SOD activities of SRI6 and SRI110 were negligible compared with FeTMPyP⁵⁺ as determined by their capacity to inhibit luminol oxidation by xanthine/xanthine oxidase-derived superoxide (Fig. 8C).

Oral delivery of SO-sparing PNDCs blocks the development of paclitaxel-induced neuropathic pain

Oral administration of SRI6 (Fig. 9A,B) and SRI110 (Fig. 9C,D) at 30 mg · kg⁻¹ · d⁻¹ for 15 d (*n* = 5) blocked the development of paclitaxel-induced neuropathic pain. Beneficial effects were associated with decrease formation of TNF-α and IL-1β (Fig. 9E,F) and increased formation of IL-10 and IL-4 (Fig. 9G,H) in spinal cord. Doses of SRI6 and SRI110 were chosen from pilot dose-ranging experiments and used at a dose found to cause near-to-maximal reversal.

Discussion

Using a well characterized rat model of paclitaxel-induced neuropathic pain resembling the peripheral neuropathies in patients (Polomano et al., 2001), our results reveal that overt production of PN is a critical determinant of pain. Inhibition of PN formation not only blocked the development of neuropathic pain, but also reversed it. Remarkably, after discontinuing FeTMPyP⁵⁺ and MnTE-2-PyP⁵⁺, mechano-allodynia and mechano-hyperalgesia did not reemerge, suggesting a possibility for long-term prevention of pain and substantial benefit to patients undergoing chemotherapy. Neuropathological changes in the spinal cord via neuroinflammation (Ledeboer et al., 2006; Peters et al., 2007; Zhang et al., 2012) and altered glutamatergic signaling (Weng et al., 2005; Cata et al., 2006a; Zhang et al., 2012) have been reported to underlie the development of paclitaxel-induced neuropathic pain. The triggering events are not well understood, but our re-

sults support PN formation in spinal cord as one potential mechanism. To this end, neuropathic pain was associated with increased NT formation in the dorsal horn, which was blocked by MnTE-2-PyP⁵⁺. The functional role of PN at spinal sites was strengthened by the findings that intrathecal delivery of the PNDCs blocked the development of paclitaxel-induced neuropathic pain. Although these findings implicate spinal PN, we cannot exclude the effects on the dorsal root ganglion due to drug exposure to intrathecal injections (Ledeboer et al., 2007). Enzymatic sources identified in PN formation included activation of NOS isoforms and NADPH oxidase; whose products, NO and SO, provide the precursors for the biosynthesis of PN (Beckman et al., 1990). An additional source was nitration of MnSOD, which disrupts its enzymatic activity providing a "feedforward" mechanism sustaining elevated PN through elevated SO (Macmillan-Crow and Cruthirds, 2001). The fact that Cu,ZnSOD activity was unaffected is consistent with the fact that PN does not affect the catalytic activity of Cu,ZnSOD (Smith et al., 1992). Viable sources for PN production include neurons and/or activated glia, since both generate NO and SO (for review, see Salvemini et al., 2011). Pinpointing the cell population(s) *in vivo* responsible for PN production is difficult given the membrane diffusability of PN to allow post-translational nitration in adjacent cells (Ferrer-Sueta and Radi, 2009). The site(s) of PN action becomes important to understanding the underlying mechanisms, particularly in glia (i.e., astrocytes and/or microglia), which through increased formation of pro- and anti-inflammatory cytokines (for review, see Watkins et al., 2001; Milligan and Watkins, 2009) and maintenance of synaptic glutamate homeostasis (Kugler, 1993) play a role in central sensitization associated with neuropathic pain states. The contribution of as-

Figure 7. Systemic or intrathecal delivery of FeTMPyP⁵⁺ or MnTE-2-PyP⁵⁺ reverse established paclitaxel-induced neuropathic pain. **A–D**, Intraperitoneal (**A, B**; 10 mg/kg) or intrathecal (**C, D**; 1 nmol/kg) administration of FeTMPyP⁵⁺ (●) or MnTE-2-PyP⁵⁺ (■) on D16 rapidly reversed mechano-allodynia (**A, C**) and mechano-hyperalgesia (**B, D**) values. Results are mean $\pm$ SD for $n = 6$ rats and were analyzed by two-tailed, two-way repeated-measures ANOVA with Bonferroni comparisons to treatment-matched D0 or baseline values. * $p < 0.001$ for baseline versus D0; † $p < 0.001$ behavior at t versus treatment-matched baseline values.

trocytes versus microglial cells in paclitaxel-induced neuropathic pain remains controversial (Weng et al., 2005; Cata et al., 2006a, 2008; Ledebore et al., 2006; Peters et al., 2007; F. Y. Zheng et al., 2011; Zhang et al., 2012) and will be best understood as selective inhibitors are developed. Notably, minocycline, a nonselective inhibitor of both microglia (Tikka et al., 2001) and astrocytes (Raghavendra et al., 2003) activation, attenuates paclitaxel-induced mechanical hypersensitivity and downregulation of both GLAST and GLT-1 in spinal cord (Zhang et al., 2012). However, it is possible that the beneficial effects of minocycline are mediated through its antioxidant (Kraus et al., 2005) and potent PN scavenging (Schildknecht et al., 2011) properties. Our findings revealed that glial cells may be important for at least two PN-dependent mechanisms in spinal cord. First, PN regulates spinal glia-derived proinflammatory and anti-inflammatory cytokines, favoring the former and correlating with increased astrocyte-associated GFAP and microglia-associated OX-42 expression. MnTE-2-PyP⁵⁺ blocked the increases in TNF- α and IL-1 β , but enhanced anti-inflammatory IL-4 and IL-10 production. The mechanism(s) behind such regulatory function(s) are unknown. However, IL-10 and IL-4 expression are NO-dependent in other settings (Chang et al., 1997; Chen et al., 2010). Therefore, increased NOS activity alone could stimulate IL-10 and IL-4; but concurrent increases in SO from NADPH oxidase activation and/or nitration/inactivation of MnSOD may deplete NO in favor of PN formation and dampen NO-driven IL-10 and IL-4

production. It is possible that reducing SO levels through preserved endogenous MnSOD activity by removing PN or directly dismutating SO, MnTE-2-PyP⁵⁺ supports NO levels beneficial to IL-10 and IL-4 production. Second, PN regulates proteins necessary for glutamatergic homeostasis. To this end, we found that the development of neuropathic pain was associated with nitration of the glia-derived GLT-1 and GS in spinal cord, a process blocked by MnTE-2-PyP⁵⁺. GLT-1 together with GLAST, another GT largely found in astrocytes, account for >80% of glutamate reuptake and control the termination of glutamatergic signaling at these sites (Kugler, 1993). PN nitration of GLT-1/GLAST blocks their transport activity, thus increasing spinal glutamate that contributes to rapid alterations in synaptic transmission (for review, see Salvemini et al., 2011). In contradistinction to the GT regulation of extracellular glutamate homeostasis, GS is pivotal in intracellular glutamate metabolic fate and in the CNS, located mainly in astrocytes that protect neurons against excitotoxicity by removing excess ammonia and glutamate and converting them into glutamine (Kugler, 1993). PN nitrates and inactivates GS (on Tyr 160), thus maintaining neuronal excitation (for review, see Salvemini et al., 2011). Thus, by removing PN and preventing GT and GS nitration, PNDCs plausibly “reset” optimal glutamatergic neurotransmission. In addition to their inactivation, downregulation of the GT

expression, observed in spinal cords during paclitaxel-induced neuropathic pain, also contributes to alterations in glutamatergic neurotransmission (Weng et al., 2005; Cata et al., 2006a; Zhang et al., 2012). We demonstrated that in addition to their ability to block neuropathic pain, PNDCs reverse it suggesting a role for PN in its maintenance; whether mechanisms of PN in this setting are similar to those that underlie its involvement in the development of neuropathic pain is not known warranting further work.

Although our studies focused on neuropathological changes in spinal cord, we cannot exclude likely contributions of PN in the periphery. The limited ability of paclitaxel to cross the blood brain barrier (Glantz et al., 1995) suggests the possibility that neuropathological changes observed in spinal cord result from changes in primary afferent fibers (Kapadia and LaMotte, 1987; Aldskogius et al., 1999) or by spinal release of factors from injured sensory neurons (Tsuda et al., 2005), rather than by direct paclitaxel effect on spinal cord. Paclitaxel-induced peripheral sensory neuropathy is associated with neuropathological processes that include increased mitotoxicity/mitochondrial dysfunction (Flatters and Bennett, 2006; H. Zheng et al., 2010, 2011; Xiao and Bennett, 2012) and peripheral loss of intradermal end nerve fibers (Siau et al., 2006; Bennett et al., 2011). Evidence suggests paclitaxel induces mitochondrial dysfunction that results in a chronic axonal energy deficit in sensory afferents, increasing their spontaneous discharge and initiating pathways ultimately leading to activation of central sensitization mecha-

Figure 8. Evaluation of SRI6 and SRI110 activities versus MnTM-4-PyP⁵⁺, MnTE-2-PyP⁵⁺ and Ebselen. **A**, Structures of PNDCs and Ebselen. **B**, Inhibition of boronic acid oxidation assay. **C**, SOD-like activity of SRI6 (●) and SRI110 (■) is significantly less than FeTMPyP⁵⁺ (▲); $p < 0.001$, $n = 3$ at each concentration. ^aSies and Masumoto, 1997; ^bFerrer-Sueta and Radi, 2009; ^cHunt et al., 1997.

Figure 9. Oral delivery of "SO-sparing" PNDCs blocks the development of neuropathic pain. **A–D**, Compared with the vehicle group (○), paclitaxel (●) induced mechano-allodynia (**A**, **C**) and mechano-hyperalgesia (**B**, **D**) that was blocked by daily (D0–D15) oral delivery (30 mg · kg⁻¹ · d⁻¹) of SRI6 (▼; **A**, **B**) or SRI110 (◆; **C**, **D**). SRI6 (▽) or SRI110 (◇) alone did not affect withdrawal thresholds in vehicle groups (**A–D**). **E**, **F**, Compared with vehicle, paclitaxel increased TNF-α and IL-1β (**E**, **F**) that was significantly attenuated by SRI6 or SRI110 (30 mg · kg⁻¹ · d⁻¹). **G**, **H**, Compared with vehicle, paclitaxel increased anti-inflammatory IL-10 and IL-4 (**G**, **H**), which was substantially enhanced by SRI6 or SRI110 (30 mg · kg⁻¹ · d⁻¹). Results are expressed as mean ± SD for 5 animals. Behavioral data were analyzed by two-tailed, two-way repeated-measures ANOVA with Bonferroni comparisons. Data for cytokine production were analyzed by two-tailed, one-way ANOVA with Dunnett's comparisons to paclitaxel group. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ for paclitaxel versus vehicle; † $p < 0.01$ or †† $p < 0.001$ for paclitaxel versus paclitaxel + SRI6 or SRI110.

nisms that promote allodynia and hyperalgesia (Flatters and Bennett, 2006; Bennett, 2010). The triggering events leading to mitotoxicity are unknown; however, PN is a candidate since mitochondria constitute a primary locus for the intracellular formation and reactions of PN and increased formation of intra- and extramitochondrially formed PN can lead to mitochondrial dysfunction and mitotoxicity in several experimental models (Radi et al., 2002). Another possible mechanism includes peripheral increase in circulating cytokines such as TNF-α that migrate across the blood brain barrier; promoting inflammation and nitroxidative stress in spinal cord. In breast cancer patients, paclitaxel has been shown to increase plasma levels of several cytokines (Tsavaris et al., 2002). Moreover, adriamycin, which does not cross the blood brain barrier, elicits inflammation and nitroxidative stress in the brain via increased plasma [TNF-α]; which is

attenuated by systemic administration of antioxidants (Joshi et al., 2010).

While pharmacological probes such as FeTM₄PyP⁵⁺ and MnTE-2-PyP⁵⁺ have excellent catalytic activities toward decomposing SO and PN, they are not orally bioavailable and may thus have limited therapeutic potential for chronic pain states. In addition, they are equally reactive with both SO and PN, which may interfere with some of the important physiological roles of SO (Massaad and Klann, 2010). To address these problems, we have retooled these catalysts for PNDC selectivity and oral activity (Rausaria et al., 2011a,b). SRI6 and SRI110, when given orally, blocked neuropathic pain development and reduced proinflammatory cytokine production, while enhancing anti-inflammatory cytokine production. Further, by virtue of this new design, SRI6 and SRI110 are significantly harder to reduce than electron

poor compounds like Mn-TM-4-PyP⁵⁺. Thus the metal-based reduction potentials of these more electron rich catalysts are out of the range useful for SOD activity (SRI6 E' < -0.2 V with +0.3 V optimal for SOD activity) (Batinic-Haberle et al., 2010), but well within range for the decomposition of PN (ONOO⁻, 2H⁺/·NO₂ E' = 1.4 V and ONOO⁻, 2H⁺/NO₂ E' = 1.2 V) (Koppenol et al., 1992). Our findings provide critical information necessary for confirming PN as a new therapeutic target for attenuating a major dose-limiting toxicity of paclitaxel without impeding on its beneficial antitumor effects.

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