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Polyester nanoparticle encapsulation mitigates paclitaxel-induced peripheral neuropathy

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Abstract

Chemotherapy utilizing cytotoxic drugs, such as paclitaxel (PTX), is still a commonly used therapeutic approach to treat both localized and metastasized cancers. Unlike traditional regimens in which PTX is administered at the maximum tolerated dose (MTD), alternative regimen like metronomic dosing (MET) are beneficial by administering PTX more frequently and in much lower doses exploiting anti-angiogenic and immunomodulatory effects. However, PTX-induced peripheral neuropathy (PIPN) and lack of patient compliant dosage forms of PTX are major roadblocks for the successful implementation of metronomic regimens. Because of the success of polyester nanoparticle drug delivery, we explored the potential of nanoparticle-encapsulated paclitaxel (nPTX) in alleviating PIPN using a rat model. Rats were injected intraperitoneally with 2 mg/kg body weight of PTX or nPTX on four alternate days and PIPN was characterized using behavioral assessments, histology and immunohistochemistry. The reduction in the tactile and nociceptive pressure thresholds was significantly less in nPTX-treated rats than in PTX-treated rats over a 16-day study period. Histological analysis showed that the degree of dorsal root ganglion (DRG) degeneration and reduction in motor neurons in the spinal cord was significantly lower in the nPTX group than the PTX group. Further, immunofluorescence data reveals that nPTX-treated rats had an increased density of a neuronal marker, β -tubulin-III (TUBB3), reduced TUNEL positive cells and increased high molecular weight neurofilament (NFH) in the spinal cord, DRG and sciatic nerves compared with PTX-treated rats. Therefore, this work has important implications in improving risk-benefit profile of PTX, paving way for metronomic regimens.

Abstract Graphic

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Author Contributions

RG, MG, MA conducted the experiments and the manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Keywords

Dorsal root ganglion; motor neurons; nanoparticles; necrosis; paclitaxel; neuropathic pain; sustained release

Introduction

The use of cytotoxic drugs for direct killing of cancer cells has dominated the field of oncology^{1,2}. Taxanes form an important class of cytotoxic agents used in the treatment of a wide range of cancers^{3–5}. Taxanes are known to block cell cycle progression through centrosome impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics leading to apoptosis^{6–10}. Generally, taxanes are administered to toxic levels reaching the maximum tolerated dose (MTD), where benefits are expected to outweigh the side effects¹¹. Recently, taxanes in combination with biologic agents are considered to broaden the range of treatment options to delay disease progression for as long as possible^{12,13}. One of the major limitations of using microtubule-targeted agents such as taxanes is the high rate of peripheral neuropathy induced by these compounds^{14,15}. Improved versions of paclitaxel (PTX) such as nab-Paclitaxel (Abraxane®), a protein bound PTX has overcome the peripheral neuropathy partially, compared to conventional solvent-based PTX¹⁶. Approaches such as the use of probiotics to counteract chemotherapy-induced neuropathy are being explored¹⁷. However, such approaches need to be evaluated in cancer models ensuring the combination does not compromise the efficacy¹⁸. On the other hand, there are currently no efficacious treatments in preventing PTX-induced peripheral neuropathy (PIPN), including those that are traditionally used in treating neuropathic pain¹⁹. Very recently, a phase IIA trial of acupuncture was shown to be safe and effective in reducing the incidence of PIPN, although, further investigations are needed to establish definitive efficacy of such approaches²⁰. In another recent clinical study with 40 breast cancer patients, cryotherapy, where patients wore frozen gloves and socks for 90 minutes that showed promising results in preventing PIPN²¹. Successful attempts were being made in developing novel non-invasive pain modifying techniques such as “Scrambler Therapy” that is aimed at re-organizing maladaptive signaling pathways by utilizing transcutaneous electrical stimulation of pain fibers²². Clinical studies are also focused on establishing the

correlation of plasma concentration and PIPN in patients receiving weekly PTX for breast cancer²³, with a view to establish better dose-regimens. There is a significant body of preclinical work attempting to understand the mechanisms of PIPN and using this as a model to discover drugs for treating pain^{24–33}. Since taxanes are neither specific nor targeted to the cancer cells, significant research has focused on improved delivery of these drugs to tumor tissues with limited success³⁴. Significant efforts are in place to develop next generation PTX formulations in the form of targeted nanoparticles that are aimed at delivering drugs directly to the tumor cells^{35–38}. While there is not much clinical success with targeted delivery of cytotoxic drugs aided by nanoparticles, some degree of success is reported for polymer micelles loaded with PTX (Genexol-PM) with an objective to increase MTD compared to conventional PTX^{39–41}. However, there are ample reports suggesting that cancer-associated fibroblasts (CAFs) responds to MTD chemotherapy and regulate tumor initiating cells (TIC) that are intrinsically more resistant to therapy^{42–44}.

Recently, metronomic chemotherapy (MET) regimens with anticancer agents is gaining significant interest for their antiangiogenic property and the potential to overcome resistance^{42,45–47}. Most importantly MET regimens prevent therapy-induced stromal activation and induction of TICs⁴². In particular, MET regimens are highly successful in the clinic for drugs that are orally bioavailable, e.g., cyclophosphamide, methotrexate, and vinorelbine^{48–51}. There is equal interest in MET regimens in veterinary setting^{52,53}. Over the years, we have been interested in formulating oral dose regimens of potent anticancer agents such as PTX, and doxorubicin (DOX) with well-established pharmacology and safety profiles with a long-term goal of establishing MET regimens with these agents^{54–56}. In the present study, we used PTX with established therapeutic efficacy on MTD regimens in rodent models of cancer to determine if re-formulating strategies (nPTX) can overcome PIPN in healthy rodents that allow nPTX to be further developed as MET regimens.

To our knowledge, this is the first study to examine the potential of polyester nanoparticle-encapsulated PTX in overcoming PIPN. Our prior studies indicate that rats injected intraperitoneal with 2 mg/kg of PTX on 4 alternate days induce profound painful PIPN^{30–32}, and we adapted similar study design and compared with PTX encapsulated nanoparticles (nPTX) at same dose and frequency. The extent of PIPN in PTX and nPTX groups was examined by measuring the degree of mechanical hyperalgesia and tactile allodynia in the hindpaws of the rats, histology of dorsal root ganglions (DRGs) and spinal cord as well as key molecular markers such as β -tubulin III (TUBB3), high molecular weight neurofilament protein (NFH) in DRG, spinal cord and sciatic nerve by immunofluorescence.

Results and Discussion

PTX encapsulated nanoparticles (nPTX) preparation and characterization

The process for making nPTX and their drug void particles have been thoroughly optimized at 500 mg scale levels. The process led to spherical particles of size 262 ± 18 nm with a polydispersity index of ~ 0.1 across 5 different batches prepared with an entrapment of 15 ± 2 mg of PTX (Figure 1a–c), while drug void particles are 252 nm. Earlier, we have shown efficacy of oral nPTX in cancer models^{53,54} and safety of the polyester particles as such on several occasions^{57,58}. These polyesters are biodegradable with established elimination

pathways via Krebs cycle that have been in human use for several decades and the outcomes of this PIPN study will allow better optimization of oral MET regimens in cancer models.

Nociceptive behavioral tests

The nociceptive behavioral tests based on tactile sensitivity (von Frey filaments) and nociceptive pressure threshold (Randall-Selitto test) measurements were conducted using the two groups of animals as described in the Methods section. There was no mortality observed in any of the groups during the course of the experiment. Systemic injection of nPTX or PTX on four alternate days (total cumulative dose of 8 mg/kg) caused a gradual decrease in the baseline tactile and pressure withdrawal thresholds in rats (Figure 2a). A significant reduction in paw withdrawal threshold (15.03 ± 5.3 g) to von Frey filaments observed in all rats treated with nPTX compared to that of PTX group (4.81 ± 1.4 g) from day 8 onwards, and the reduction in the paw withdrawal thresholds persisted for at least another 10 days after the last treatment, until day 16 (Figure 2a). Unlike PTX group (12.42 ± 4.11 g), the nPTX group (20.42 ± 3.2 g) did not differ significantly from baseline values (nPTX: 25.43 ± 3.5 g; vs. PTX: 25.61 ± 4.5 g) until day 6. However, both groups showed reduced tactile withdrawal threshold on day 8 and stayed at respective values until day 16 (Figure 2a).

Similarly, the mean paw withdrawal threshold in response to the noxious pressure stimulus was reduced markedly from 197 ± 15.6 g to 132 ± 13.9 g in PTX group. By comparison, there was a small reduction in the pressure threshold in nPTX group, from 213 ± 35.3 g to 178 ± 23.68 g (Figure 2b). PIPN is considered as a major dose-limiting problem of PTX therapy. However, we are not ready yet to consider exposure-guided dosing regimens for improving risk-benefit profiles of PTX²³. A primary reason for this could be due to heavy reliance on MTD regimens aiming at killing cancer cells and such high levels of systemic PTX can expose non-target vulnerable cell bodies such as DRG that lacks efficient vascular barrier⁵⁹. Taking cues from our earlier efficacy data with PTX in cancer models⁵⁴ as well as other studies using a variety of drugs with established toxicity profiles and their success with reformulation strategies⁶⁰, we have designed this study to compare PTX with nPTX head on for its ability to overcome PIPN. The reformulation strategies permit substantial dose reduction, almost by 50% without compromising the efficacy^{54,60}, that is primarily driven by modified pharmacokinetic profiling of the encapsulated drug^{54,60,61}. In this study, nPTX offered better performance presumably due to sustained release behavior as opposed to PTX that is readily exposed to tissues making rats sensitive to pain (Figure 2a, b). Such risk-benefit profile studies should be better done in animal models of human pathology, in this case, cancer; however, behavior studies in such models could be complicated. These behavior analyses are further corroborated with histology and immunofluorescence, so that a correlation is established that can be taken further to efficacy studies where risk-benefit profiles can be established.

Histopathology and immunofluorescence

The dorsal root ganglion (DRG) is a highly vulnerable site and is involved in pathophysiological conditions such as pain and PTX is known to affect this process by increased spontaneous activity (SA) in DRG neurons^{62,63}. PTX-induced neuronal death in DRGs is due to necrosis that is regulated by activating transcription factor 3 (ATF3)^{64, 65}. In

the current study, a significant decrease in the DRG neurons was observed in PTX groups, while nPTX showed high numbers (Figure 3a, b). The neurons in DRGs from nPTX rats are round or oval with centrally placed vesicular nucleus and prominent nucleolus. Also, seen are neurons surrounded by many satellite cells and perikaryon containing Nissl substance. However, the DRGs from PTX, revealed shrinking cells, blurry nuclei, a decrease of the Nissl bodies and widening of the cell gaps (Figure 3a). It is interesting to note that the concentration of PTX in the DRGs is same in both the groups (Figure 3c), though nPTX mitigates the PIPN. This is probably because the PTX concentration presented is the sum of free and entrapped PTX that is difficult to separate⁶⁶. The nPTX offers sustained release over a period, predominantly by diffusion-based mechanism as the polyester hydrolyzes, limiting the peak concentrations (C_{max}) that has been considered one of the primary reasons for PTX-induced neuropathy^{23,54,55,61}. Prior literature suggests that PIPN is due to degeneration of afferent sensory axons that have the ability to regenerate, however, the degree and reversibility is dose and duration dependent⁶⁷.

It is well perceived that the sensory neurons are more sensitive to PTX compared to motor neurons, making motor neuropathy less frequent^{68,69}. This is because the cell bodies of sensory neurons lie outside the blood brain barrier, exposing them to higher levels of PTX than those of motor neurons within the spinal cord⁵⁹. This reflects from the tissue distribution data, where PTX levels between both groups are statistically insignificant and are about 100 orders of magnitude lower than DRG levels (Figure 4c). Consistent with behavior assessment and DRG histology, the rats in nPTX group showed higher number of motor neurons in spinal cord compared to PTX group (Figure 4b). We also observed axons and oligodendrocytes degeneration in PTX group and not nPTX (Figure 4a). Our earlier studies suggests that PTX treatment increases presynaptic metabotropic glutamate receptor 5 (mGluR5) activity at spinal cord level that serves as upstream signaling for protein kinase C (PKC)-mediated tonic activation of presynaptic N-methyl-D-aspartate receptors (NMDARs), leading to increased nociceptive input from primary afferent nerves and development of neuropathic pain^{30,31}. Unlike, peripheral sensory neurons, spinal cord neurons are unable to regenerate^{67,70} and our data suggests that PTX causes neuronal death and this could be addressed in part by developing sustained release formulations (Figure 4a, b).

The microtubule stabilizing drug PTX is widely used as a cytostatic agent in oncology at maximum tolerated doses (MTD); however, its application is limited by the risk of dose dependent neuropathies. On the other hand, at low doses, it can stabilize the microtubules and prevent axon degeneration and promote survival^{71,72}. The microtubules are integral to healthy functioning of axons and tubulin β -III (TUBB3) is the most dynamic β -tubulin isoform that is highly expressed in axons⁷³. The degeneration of axons interrupt the signal flow from one neuron to the other and been identified as major pathology in PIPN. A recent report suggests that TUBB3 is not required for normal neuronal function; however, it is necessary for timely axon regeneration⁷⁴. Our TUBB3 immunofluorescence data shows significantly lower staining in PTX group compared to nPTX across all the tissues suggesting axons are degenerated (Figure 5a–c). The morphometric analysis reveals that the DRGs in PTX group are shrunken and lost the sheath around neuronal cell body, while in the nPTX group we can see a thick sheath around the DRG (Figure 5b). Similarly, in PTX group we see significant axon degeneration in the sciatic nerve that is made up of axons of sensory

and motor neurons (Figure 5c). The TUBB3 data is consistent with the histology findings and behavior outcomes. There is ample literature suggesting that moderate microtubule stabilization decrease scarring and enables axon regeneration after spinal cord injury⁷⁵. Overall, these findings suggest that in PTX group the breakdown of the microtubule cytoskeleton leads to axon degeneration contributing to PIPN. The role of calcium signaling in peripheral neuropathy is well documented in the literature and parvalbumin (PVALB) is the most commonly used neuronal marker⁷⁶. A recent study proposes that dorsal horn PVALB interneurons function as gate-keepers of touch-evoked pain after nerve injury. In doing so, they demonstrated that ablating PVALB neurons in naive mice produce neuropathic pain-like mechanical allodynia via disinhibition of PKC γ excitatory interneurons. In contrary, activating PVALB neurons in nerve-injured mice alleviates mechanical hypersensitivity⁷⁷. PVALB immunoreactivity was insignificant between the two groups in spinal cord (dorsal/ventral horns and grey matter areas) and sciatic nerve but significant in DRGs (Figure 5a–c). The TUBB3/PVALB colocalization is significant between PTX and nPTX, however the trends are reverse where spinal cord and sciatic showed on the lower side, while DRG is high and this is in alignment with high TUBB3 and significant/insignificant PVALB staining (Figure 5a–c). The PVALB data from this study is inconclusive because both injured and uninjured neurons can stain positive without having to downregulate in injured and upregulate in spared neurons⁷⁸ can stain positive without having to downregulate in injured and upregulate in spared neurons⁷⁸. Further investigations with more specific neuronal marker such as activating transcription factor 3 (ATF3) to identify injured neurons may help understand the role of PVALB in PIPN.

PTX treatment increases terminal dUTP nick end labeling (TUNEL)-positive necrotic astrocytes, DRG neurons, and sciatic nerves, however these neurons stay protected in nPTX group with no TUNEL-positive nuclei (Figure 6). A plausible mechanism of PTX peripheral neuropathy is via the activation of spinal astrocytes that can be evaluated by the expression of GFAP⁷⁹. The expression intensity of GFAP in the PTX-treated group appears significantly lower than the nPTX group, probably due to the fact that the TUNEL positive nuclei are very high in PTX group masking the GFAP impact (Figure 6a). Further, our data is consistent with the literature⁸⁰ that PTX treatment would lead to DRG necrosis leading to allodynia that minimizes in nPTX group (Figure 6b). The immunohistochemical analysis showed that both PTX and nPTX administration did not alter the GFAP immunoreactivity in the DRGs (Figure 6b). Sciatic nerves showed loss of GFAP staining in PTX group due to loss/breaks of axons/nerve fibers; however, one can see long axons in the nPTX group (Figure 6c). The axonal loss in PTX group reflected in the behavior study making rodents sensitive to pain, probably due to inefficient nerve regeneration as the breaks in fibers lead to disconnect between Schwann cells, axons and the extracellular matrix.

Myelin is an insulator and forms an important component of vertebrate system that envelopes most of nerve cell axons. An enzyme called 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) makes up a large part of total protein within the non-compact myelin regions, and its dysfunction causes severe neurological symptoms. The lack of CNPase can lead to loss of axons and severe neurological complications⁸¹, and overexpression of CNPase can lead to a reduction in the overall amount of myelin, as well as lead to inflammation⁸². We have observed highly significant CNPase staining in both central

(oligodendrocytes) and peripheral (Schwann cells) tissues in PTX groups, but not in nPTX groups (Figures 7a–c). In contrast, PTX drastically decreased high molecular weight neurofilament (NF-H) that is essential in axonal stabilization, which is preserved in nPTX (Figure 7a–c). In nPTX group, we see a strong co-localization of CNPase and NF-H, presenting an intact central and peripheral nervous system that corroborates with behavior data (Figure 7a–c).

Conclusion

We have shown that PTX-encapsulated polyester nanoparticles with established therapeutic potential in cancer models, mitigates PTX-induced peripheral neuropathy. Our findings suggest that sustained release MET dose regimens is a promising strategy to maximize therapeutics benefits minimizing PIPN. These findings need to be verified in tumor models on MET regimens in order to establish risk-benefit profile.

Methods

PTX encapsulated nanoparticles (nPTX) preparation and characterization

PTX encapsulated nanoparticles are prepared according to protocols previously developed in our lab with slight modifications^{54,55}. In brief, 500 mg polylactide-coglycolide [PLGA (50:50) Resomer R503H; MW 35–40 kDa] dissolved in 20 mL of ethyl acetate, and 30 mg of PTX dissolved in 5 mL of ethyl acetate, under stirring for 1 h @1000 rpm, constitutes the organic phase and this is further stirred for 15 min. Aqueous phase is prepared by dissolving 750 mg of d- α -Tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS) in water under stirring for 1 h at 1000 rpm. The oil phase is added to aqueous phase dropwise and stirred for 30 min at 1000 rpm. This emulsion is subjected to homogenization for 45 min at 10,000 rpm. The emulsion is added to 250 mL of water to facilitate the diffusion of organic solvent to aqueous phase that is then evaporated overnight under stirring at 1000 rpm. The particle size is measured for fresh preparations (20 μ L sample diluted to 1 mL using water) using zeta sizer (Malvern Nano ZS). The total preparation is about 300 mL that is distributed equally into centrifuge tubes and centrifuged at 15,000 g/4 °C for 30 min. The supernatant is collected and stored for analyzing the free PTX. To the pellets, 25 mL of sucrose solution (5% w/v) added and pooled together. This suspension is distributed 10 mL into 20 mL vials and 2 mL each in 5 mL vials for particle size and entrapment efficiency post freeze drying. The freeze drying conditions are as follow: 1) Freeze (3 h) –50 °C; safety pressure 1.650; 2) main drying (48 h) –50 °C; 0.004 mBar vacuum; safety pressure 1.650; 3) final drying +20 °C; 0.004 mBar vacuum for 12 h; safety pressure 1.650. The freeze dried samples (2 mL volume samples) are re-constituted in 40 mL water by probe sonicating for 30 sec @ 20% amplitude and particle size is recorded. The samples are subjected to centrifugation to collect the pellet at 18,000 g/4 °C for 30 min. The pellets are dissolved in acetonitrile and entrapment is assessed by HPLC method reported earlier^{54,55}.

Animal Model and PTX/nPTX Treatment

To assess if nanoparticle encapsulated PTX (nPTX) can mitigate PTX-induced peripheral neuropathy, experiments were conducted using adult male Sprague-Dawley rats (220–250 g;

Harlan, Indianapolis, IN). A total of 16 rats (n=8 per group) was used for the entire study. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats received 2 mg/kg PTX (cremophor EL used as a vehicle) or nPTX (water used as a vehicle) intraperitoneally on four alternate days, as described previously ^{8,31}. We confirmed the presence of mechanical hyperalgesia and tactile allodynia in the hindpaws of the rats 8 to 16 days after the completion of PTX treatment. All terminal experiments were conducted 16 days after the last PTX or nPTX injection.

Nociceptive behavioral tests

To determine tactile sensitivity, individual rats were acclimatized for 30 min on a mesh floor within suspended chambers. Once acclimatized, each rat was applied a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) perpendicularly to the plantar surface of both hind paws with sufficient force to bend the filament for 6 s and the response was recorded. A brisk withdrawal or flinching of the paw was considered a positive response. In the absence of a response, a filament of the next greater force was applied. If a response was observed, a filament of the next lower force was applied. The tactile stimulus with a 50% likelihood of producing a withdrawal response was calculated using the “up-down” method⁵⁶.

To determine the mechanical nociceptive threshold, we conducted the paw pressure (Randall-Selitto) test on the left hind paw using an analgesimeter (Ugo Basile, Varese, Italy). A constantly increasing force on a linear scale was applied to the hind-paw. When the animal displayed pain by withdrawing the paw or vocalizing, the device was immediately inactivated, and the animal's withdrawal threshold was read on the scale^{83, 84}. A maximum of 400 g of pressure was used as a cutoff to avoid tissue injury to the rats. The investigator conducting the behavioral tests was blinded to the treatment.

Histology and immunofluorescence studies

After the last dose of treatment on the 16th day, terminal anesthesia was induced by administering 0.9 ml of 3 mg/ml pentobarbitone. Subsequently, transcardiac perfusion with 60 ml of 0.9% NaCl followed by 60 ml of 4% paraformaldehyde in 0.1 M phosphate buffer was carried out. L2–L5 dorsal root ganglions (DRGs), sciatic nerves (SN) and spinal cord (SC) at mid-thigh were carefully dissected from each animal, fixed in 4% paraformaldehyde until the tissues were properly fixed. After fixation, the DRG, SN, SC tissues were processed for paraffin embedding. Each tissue was sectioned at a thickness of 6 μ m onto charged slides that were then stored at –80°C. General H&E staining were performed for all sections. Images were acquired with a bright-field microscope at the original magnification of 10 $\times$ and 40 $\times$ (3000-LED Microscope; ACCU-SCOPE). Quantitative analyses were performed using multiple images (10 different fields of view at 10 $\times$ magnification per slide) from multiple rat samples (each slide containing multiple SC, DRG, or SN 6–8 tissue sections) under the microscope. For immunofluorescence, tissue slides were warmed to 60 degrees Celsius, washed sequentially in xylene, 90% ethanol, and PBS solutions (each for 10 minutes). To prevent non-specific binding, the slides were blocked for 1 hour in PBS containing 3% normal goat serum or horse serum (Vector Laboratories Inc., USA). The

slides were incubated overnight in a humidity chamber with the following antibody pairs: rabbit monoclonal antibody to β 3-Tubulin (D71G9 clone; 1:200; 5568, Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse monoclonal antibody to Parvalbumin (PARV-19 clone; 1:1000; Sigma, USA), or rabbit monoclonal antibody to CNPase (D83E10 clone; 1:100; Cell Signaling # 5664, USA) and mouse monoclonal antibody to neurofilament-H subunit (RNF402 clone; 1:50; Santa Cruz –32729, USA). Slides were then washed and incubated with respective anti-mouse (Alexa Fluor 488, goat anti-mouse) or anti-rabbit (Alexa Fluor 594 goat anti-rabbit) secondary antibodies (1:2000; Invitrogen technologies, Carlsbad, CA, USA) for 2 hours. For dual labelling of GFAP and TUNEL, the SC, DRG, SN sections were stained with Rabbit monoclonal antibody to GFAP (D1F4Q clone; 1:200; Cell Signaling Technology #12389, Danvers, MA, USA) overnight in the humidity chamber. The slides were washed and incubated with an anti-rabbit (Alexa Fluor 594) secondary antibody (1:2000; Invitrogen technologies, Carlsbad, CA, USA) for 2 hours. The slides were washed three times with PBS, and the tissue sections were stained with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture using an *in situ* cell death detection kit (Roche, Mannheim, Germany). Finally, the slides were washed and cover-slipped with Vectashield Antifade Mounting Media containing DAPI (Vector laboratories, Burlingame, CA). Tissue sections were imaged using a confocal laser scanning microscope (Zeiss LSM 780, Carl Zeiss Microscopy, Jena, Germany) at 20 $\times$ original magnification, and in case of SC, whole composite images were acquired at 6 $\times$ magnification under tile scan mode (3 $\times$ 3). Fluorescence quantification was performed with ImageJ software and the results were expressed as an average of at least 10–14 images per group. Fluorescent image analysis was performed with a Zeiss ZEN Software (Zen Blue 2.3).

PTX levels in DRG and spinal cord

In a separate study, we used 4 rats to assess the distribution of PTX in the DRG and SC tissues. The rats weighing 220–250 g (Harlan, Indianapolis, IN) were administered 2 mg/kg intraperitoneally in a similar regimen as used in the PINP study, but the rats were sacrificed 2 h after injecting the fourth dose of PTX/nPTX and the DRG and SC were collected for PTX estimation.

LC-MS method for PTX analysis in DRG and SC

The PTX in samples were detected and quantified on a triple quadrupole mass spectrometer (Quantiva, Thermo Scientific, Waltham, MA) coupled to a binary pump HPLC (UltiMate 3000, Thermo Scientific). MS parameters were optimized for PTX under direct infusion at 5 μ L min⁻¹ to identify the SRM transitions (precursor/product fragment ion pair) with the highest intensity as 876.3–308.04 m/z for the sodium adduct of PTX and 830.3–549.2 m/z for the internal standard, Docetaxel. Samples were maintained at 4 $^{\circ}$ C on an autosampler before injection. The injection volume was 10 μ L. Chromatographic separation was achieved on a Hypersil Gold 5 μ m 50 $\times$ 3 mm column (Thermo Scientific) maintained at 30 $^{\circ}$ C using a solvent gradient method. Solvent A was water (0.1% formic acid). Solvent B was acetonitrile (0.1% formic acid). The gradient method used was 0–1.6 min (20% B to 80% B), 1.6–4 min (80% B), 4–5 min (80% B to 20% B), 5–6 min (20% B). The flow rate was

0.5 mL min⁻¹. Sample acquisition and analysis was performed with TraceFinder 3.3 (Thermo Scientific). Data is calculated using standard curves generated for PTX.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 Software. Data were presented as the means S.E. or S.D. To compare the difference in the pain hypersensitivity and tissue data between PTX and nPTX-treated groups, we used twoway analysis of variance (ANOVA) followed by Tukey's multiple comparison test (figure 2) or Student's t-test (figures 3, 4, 5, 6 and 7). The level of statistical significance was set at $*p < 0.05$.

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Figure 1.

The characteristics of nPTX used in this present study. a) Table showing the formulation characteristics of different batches b) Representative dynamic light scattering (DLS) particle size distribution profile and c) Representative scanning electron micrograph of nPTX. *The difference in the entrapment numbers is not the reproducibility issue, but different volume of the suspension used in the vial for freeze drying.

Figure 2.

nPTX reduces the pain hypersensitivity of rats compared to those treated with PTX. *a)* Tactile threshold and *b)* Pressure threshold, time course of the effect of intraperitoneal injections of PTX and nPTX at 2mg/kg administered as indicated by arrows on x-axis. BL is baseline data. We used same rats in a & b experiments. We used same rats in a & b experiments. Comparisons were made between PTX vs nPTX using two-way analysis of variance followed by Tukey's multiple comparisons test ($n=8$) Error bars represent the S.E. [*a)* day 6 *** $p < 0.001$; day 8 **** $p < 0.0001$; day 10 **** $p < 0.0001$; day 12 *** $p < 0.001$; day 14 **** $p < 0.0001$; day 16 *** $p < 0.001$. *b)* day 4 ** $p < 0.01$; day 6 *** $p < 0.001$; day 8 **** $p < 0.0001$; day 10 *** $p < 0.001$; day 12 ** $p < 0.01$; day 14 ** $p < 0.01$; day 16 *** $p < 0.001$].

Figure 3. nPTX prevents dorsal root ganglion (DRG) degeneration. *a)* H&E-stained sections were prepared from the DRG nerve at the end of the study. *b)* PTX group showed degeneration of neurons presenting significantly low in DRG numbers compared to nPTX group indicated with bright red arrowheads. *c)* PTX levels in DRG, the difference between PTX and nPTX groups is not statistically significant ($n=4$). Error bars represent the S.D., 8–10 images were used and analysis was performed using Student's *t*-test, *** $p < 0.001$.

Figure 4.

nPTX increase motor neuron survival in spinal cord (SC) region. *a)* Representative images of ventral root horn spinal cords of PTX and nPTX treated rats. *b)* SC motor neuron counts significantly decreased in PTX groups compared to nPTX. Images were acquired at 10 and 40 $\times$ original magnification. *c)* PTX levels in SC, the difference between PTX and nPTX groups is not statistically significant ($n=4$). The PTX levels between DRG and SC are about 100 orders of magnitude, presumably due to the deficiency in blood-nerve barrier in the former. The yellow arrows mark axons and red are oligodendrocytes. Error bars represent the S.D., 8–10 images were used and analysis was performed using Student's *t*-test, *** $p < 0.001$.

Figure 5.

Double immunofluorescence with β -tubulin III [TUBB3] (red) and parvalbumin [PVALB] (green) in **a)** spinal cord showing high TUBB3 and insignificant PVALB staining in nPTX compared to PTX group, images are acquired at 6 $\times$ magnification under tile scan mode (3 $\times$ 3) **b)** DRG showing high TUBB3 and PVALB staining in nPTX compared to PTX group, yellow arrow marks myelin sheath in nPTX group that is absent in PTX **c)** sciatic nerve showing high TUBB3 and insignificant PVALB staining in nPTX compared to PTX group. Quantification of TUBB3, PVALB and TUBB3/PVALB colocalization in **a)** spinal cord **b)** DRG and **c)** sciatic nerve. Error bars represent the S.D., 8–10 images were used and analysis was performed using Student's t test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 6.

Double immunofluorescence with GFAP (red) and TUNEL (green) in *a*) spinal cord showing high TUNEL positive cells and low GFAP staining in PTX compared to nPTX group, images are acquired at 6× magnification under tile scan mode (3×3) *b*) DRG showing high TUNEL positive cells and insignificant GFAP staining in PTX compared to nPTX group *c*) sciatic nerve showing high TUNEL positive cells and low GFAP staining in PTX compared to nPTX group. Quantification of TUNEL positive cells and GFAP in *a*) spinal cord *b*) DRG and *c*) sciatic nerve. Error bars represent the S.D., 8–10 images were used and

analysis was performed using Student's t test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

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Figure 7.

Double immunofluorescence with CNPase (red) and NF-H (green) in *a*) spinal cord showing high CNPase and low NF-H staining in PTX compared to nPTX group, images are acquired at 6x magnification under tile scan mode (3x3) *b*) DRG showing high CNPase and low NF-H staining in PTX compared to nPTX group *c*) sciatic nerve showing high CNPase and low NF-H staining in PTX compared to nPTX group. High magnification images of *a1*) spinal cord *b1*) DRG and *c1*) sciatic nerve. The CNPase and NF-H fluorescent intensities as well as CNPase/NF-H %colocalization for all the tissues were plotted. Error bars represent the S.D., 8–10 images were used and analysis was performed using Student's t test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.