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Prevention of chemotherapy-induced peripheral neuropathy by the small-molecule inhibitor Pifithrin-μ

Karen Krukowski[1], **Cora H. Nijboer**[2], **XiaoJiao Huo**[1], **Annemieke Kavelaars**[1], and **Cobi J. Heijnen**[1,*]

[1]Laboratory of Neuroimmunology, Department Symptom Research, Division of Internal Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, USA [2]Laboratory of Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht, Utrecht, the Netherlands

Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect of cancer treatment. CIPN is the most frequent cause of dose reduction or treatment discontinuation in patients treated for cancer with commonly used drugs including taxanes and platinum-based compounds. No FDA-approved treatments for CIPN are available. In rodents, CIPN is represented by peripheral mechanical allodynia in association with retraction of intraepidermal nerve fibers (IENFs). The mechanism of chemotherapy-induced neurotoxicity is unclear but it has been established that mitochondrial dysfunction is an important component of the dysregulation in peripheral sensory neurons.

We have shown earlier that inhibition of mitochondrial p53 accumulation with the small compound pifithrin-μ (PFT-μ) prevents cerebral neuronal death in a rodent model of hypoxic-ischemic brain damage. We now explore whether PFT-μ is capable of preventing neuronal mitochondrial damage and CIPN in mice. We demonstrate for the first time that PFT-μ prevents both paclitaxel- and cisplatin-induced mechanical allodynia. Electron microscopic analysis of peripheral sensory nerves revealed that PFT-μ secured mitochondrial integrity in paclitaxel-treated mice. In addition, PFT-μ administration protects against chemotherapy-induced loss of intra-epidermal nerve fibers (IENF) in the paw. To determine whether neuroprotective treatment with PFT-μ would interfere with the anti-tumor effects of chemotherapy, ovarian tumor cells were cultured *in vitro* with PFT-μ and paclitaxel. PFT-μ does not inhibit tumor cell death but even enhances paclitaxel-induced tumor cell death. These data are the first to identify PFT-μ as a potential therapeutic strategy for prevention of CIPN to combat one of the most devastating side effects of chemotherapy.

*Correspondence: Dr. Cobi J. Heijnen, Laboratory of Neuroimmunology, Department Symptom Research, Division of Internal Medicine, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Unit 384, Houston, TX, USA, CJHeijnen@mdanderson.org.

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Keywords

chemotherapy; neuropathy; pifithrin-μ; mitochondria; peripheral nerves

Introduction

The American Cancer Society estimates 1.6 million people will be diagnosed with cancer in 2014 and nearly 14.5 million cancer survivors live in the U.S. today. Of these newly diagnosed cases and cancer survivors nearly 60% will experience pain, tingling, and numbness in hands and feet due to the side effects of chemotherapy [9; 32; 33; 47–49]. This chemotherapy-induced peripheral neuropathy (CIPN) is the major dose-limiting toxicity associated with cancer treatment. The highest incidence of CIPN is associated with taxanes (paclitaxel) and platinum-based (e.g. cisplatin) treatment [32; 33; 47]. With no FDA-approved drugs to prevent or treat CIPN, a significant number of cancer patients receive sub-optimal anti-cancer treatment due to dose reduction needed because of neuropathic pain. In addition, CIPN frequently persists long after completion of chemotherapy thereby reducing quality of life of cancer survivors [45]. Furthermore, new anti-cancer treatments frequently used in combination with chemotherapy can exacerbate symptoms of CIPN. Vascular endothelial growth factor (VEGF) inhibitors (bevacizumab, sorafenib and sunitinib) as well as immune modulating compounds (thalidomide) have been reported to increase incidence of CIPN which can often leads to early treatment cessation[23; 41; 44].

In rodent models of CIPN increased sensitivity to a mechanical stimulus (mechanical allodynia) is commonly used as a read out for pain [7; 13; 16; 27; 34; 46]. Paclitaxel stabilizes microtubules thereby inhibiting with cell division [35]. Cisplatin exerts its anti-tumor effect by binding to and crosslinking DNA which triggers cellular apoptosis. Additionally, both drugs have been shown to interfere with mitochondrial function in tumor cells as well as in non-dividing cells [2; 10; 17; 43]. In peripheral sensory neurons increased oxidative stress, excessive calcium levels, and ATP depletion [1; 11; 37; 53–55] can lead to spontaneous discharge of peripheral sensory neurons. Spontaneous discharge of both A- and C- fiber sensory neurons has been linked to neuronal sensitization and hyperalgesic responses [50–52]. One of the mechanisms that can lead to alterations in oxidative stress, calcium levels, and ATP, is improper mitochondrial function [1]. Furthermore, disruption of mitochondrial function has also been associated with a reduction in IENF density [14]. In this work we propose that prevention of chemotherapy-induced mitochondrial dysfunction may be a promising avenue for inhibition of CIPN.

The small molecule inhibitor, Pifithrin-μ (PFT-μ) has been identified for its capacity to inhibit mitochondrial p53 accumulation without impacting p53 transcriptional activity [39]. Our group demonstrated previously that disruption of the p53 mitochondrial pathway as well as intraperitoneal administration of PFT-μ protects against cerebral neuronal loss in a rodent model of neonatal ischemic brain damage [28–31]. In this model, PFT-μ prevented mitochondrial accumulation of p53 in the brain, thereby reducing oxidative stress and maintaining ATP production.

We hypothesized that protection of neuronal mitochondria by PFT-μ might also be a means to prevent CIPN. When investigating potential therapeutics for CIPN it is most critical that these drugs do not interfere with cancer therapy. Therefore, we also explored possible interference of PFT- μ with *in vitro* tumor cell killing induced by paclitaxel.

Methods

Animals

Adult female C57BL/6 mice were housed at the Texas A&M Health Science Center Program for Animal Resources. Mice were housed on a regular 12 h light/dark cycle with free access to food and water. All procedures were consistent with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the Ethical Issues of the International Association for the Study of Pain[56] and were approved by the local IACUC.

Drug Administration

Paclitaxel (6 mg/ml) in 50% El Kolipher (Sigma Aldrich, St. Louis, MO) and 50% ethanol (Sigma Aldrich) was diluted in sterile saline to final concentration of 1 mg/ml and administered i.p. at a dose of 10 mg/kg every other day for two weeks. Cisplatin (Sigma Aldrich) was diluted in sterile saline and administered i.p. at a dose of 2.3 mg/kg daily for 5 days, followed by 5 days of rest, and as second round of 5 doses of cisplatin. PFT-μ (25 mg/ml in DMSO (Sigma Aldrich)) was diluted in sterile saline. PFT-μ was administered 1 hour prior to chemotherapy treatment intraperitoneally (i.p.) at a dose of 8 mg/kg [30; 40]. PFT-μ was administered one hour prior to each dose of chemotherapy and not on any of the other days. We have shown that these chemotherapy dosing schedules induce CIPN in mice [34]. Additionally, this dosage of PFT-μ was effective in protecting rodent neuronal mitochondria in ischemia [30].

Von Frey test for mechanical allodynia

Mechanical allodynia as a readout for CIPN was measured as the hind paw withdrawal response to von Frey hair stimulation using the up-and-down method as we described previously[34; 46]. Mice were placed in a plastic cage (10×10×13 cm^3) with a mesh floor for 30 min prior to testing. Subsequently, a series of von Frey hairs (0.02, 0.07, 0.16, 0.4, 0.6, 1.0 and 1.4 g) (Stoelting, Wood Dale, Illinois, USA) were applied perpendicular to the mid-plantar surface of hind paw. A trial began with the application of the 0.16 g hair. A positive response was defined as a clear paw withdrawal or shaking. Whenever a positive response occurred, the next lower hair was applied, and whenever a negative response occurred, the next higher hair was applied. The testing consisted of five stimuli after the first change in response occurred, and the pattern of response was converted to a 50% von Frey threshold using the method described previously [6] by an investigator blinded to treatment until the end of the experiment.

Transmission Electron Microscopy for Mitochondrial Morphology

To investigate mitochondrial integrity, dorsal root ganglia (DRG) and peripheral nerve axons (sciatic nerve removed by mid-thigh removal method) were fixed in a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3. Samples

were washed in 0.1 M cacodylate buffer and treated with 0.1% Millipore-filtered buffered tannic acid, post fixed with 1% buffered osmium tetroxide for 30 min, and stained and blocked with 1% Millipore-filtered uranyl acetate. The samples were washed in water, dehydrated with increasing concentrations of ethanol, infiltrated, embedded in LX-112 medium and polymerized in a 60 C oven for 2–3 days. Ultrathin sections were cut in a Leica Ultra cut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

A blinded scorer rated mitochondria as typical or atypical. This chemotherapy-induced mitochondria rating tool was established by Flatters et al. [11]. Percentage of atypical mitochondria was calculated for each mouse (n=3). Only mitochondria fully in the field of view were scored and mitochondria under 165 nm in diameter were excluded from scoring. Atypical mitochondria were identified by 2-fold increases in diameter or excessive vacuolization (more 50% translucent). For DRG tissues 10–25 mitochondria scored/mouse, for peripheral nerve axons 7–18 mitochondria scored/mouse. Preliminary studies have shown that there are no differences in mitochondrial morphology between vehicle-alone or PFT- μalone treated mice. Therefore PFT- μ-alone treated mice were used for baseline comparisons. Furthermore, percentages of atypical mitochondria in PFT- μ alone treated mice were comparable to what has been reported by others under baseline conditions in previously published studies [54].

Immunostaining for IENFs

For quantification of IENFs, 3 mm^2 biopsies from the plantar surface of the hind paws were collected 3 weeks after completion of paclitaxel treatment) and processed as described [4; 5; 34; 38]. Biopsies were immediately placed in Zamboni's fixative for 24 hrs., transferred to 20% sucrose, frozen in Optimal Cutting Temperature compound (OCT), and sliced into 25 μm sections. The sections were blocked for 2 hrs. At room temperature in 0.1 M PBS containing 5% normal donkey serum/0.3% Triton X-100. Sections were incubated with an antibody against the pan neuronal marker PGP9.5 (AbD Serotec; Rabbit, 1:1000) along with anti-Collagen IV antibody (Southern Biotech; Goat, 1:100) followed by Alexa-594-donkey anti-rabbit (Life Technologies, 1:500) and Alexa-488-donkey anti-goat (Invitrogen, 1:500). Secondary antibodies alone did not give a signal, indicating specificity of the staining. Three randomly chosen sections from each paw were quantified under a Leica fluorescence microscope. Nerve fibers that crossed the collagen stained dermal/epidermal junction into the epidermis were counted in three fields of each slice using the 40× objective. The length of the epidermis within each field was measured using ImageJ. IENFs density was determined as the total number of fibers/length of epidermis (IENFs/mm). A scorer blinded to the experimental set up rated the IENF density.

Tumor Cell Assay

Human ovarian tumor cells (HeyA8) were a gift from Dr. Anil Sood, MD Anderson Cancer Center [12; 18]. Tumor cells were maintained in 5% CO2 at 37 °C in RPMI-1640 media plus 15% fetal bovine serum and 0.1 % antibiotics. Tumor cell viability was measured by an

enzymatic MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay. HeyA8 cells (3,000 cells /well) were seeded in flat bottom 96 well plates. After 24 hours fresh media was added to the wells +/− PFT-μ (5–20 μM) and +/− paclitaxel (15 nM) for 72 hours. After 72 hrs. MTT (0.15%) was added to each well and incubated for 2 hrs. followed by DMSO. Colorimetric changes were measured at 570 nm on a Biotek Epoch plate reader.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was carried out using repeated measure analysis of variance by two-way ANOVA or one-way ANOVA followed by Bonferroni analysis. $P < 0.05$ was considered statistically significant.

Results

The effect of PFT-μ administration on paclitaxel-induced peripheral neuropathy

To investigate if PFT-μ administration prevents paclitaxel-induced mechanical allodynia, adult C57/Bl6 mice were treated with 10 mg/kg paclitaxel +/− 8 mg/kg PFT-μ every other day for two weeks. In mice treated with paclitaxel alone, decreased paw-withdrawal thresholds were observed as measured with the von Frey test for five weeks after paclitaxel administration. This decrease in paw-withdrawal threshold is indicative of chemotherapy-induced mechanical allodynia. PFT-μ completely prevented development of paclitaxel-induced decreases in paw-withdrawal thresholds. No differences in paw-withdrawal threshold were detected between mice treated with vehicle alone, PFT-μ alone or PFT-μ + paclitaxel. These data demonstrate that systemic administration of PFT-μ completely prevents paclitaxel-induced mechanical allodynia (see Figure 1).

The effect of PFT-μ administration on mitochondrial morphology in peripheral sensory neuron cell bodies and axons

Paclitaxel induces swollen and vacuolated mitochondria in peripheral sensory neurons which may contribute to CIPN [11; 21; 26; 54]. Using transmission electron microscopy we confirmed that mitochondria in peripheral sensory nerve cell bodies in lumbar dorsal root ganglia (DRGs) and axons in the sciatic nerve of paclitaxel-treated mice were enlarged in shape, had disorganized cristae, displayed swelling/vacuolization, and loss of double membranes (denoted by white arrows) (Figure 2 and 3). Furthermore, the number of atypical mitochondria in DRGs and nerves was increased following paclitaxel treatment (Figure 2E, 3E).

Administration of PFT-μ completely prevented these paclitaxel-induced alterations in mitochondrial morphology in the DRGs (2C, D) and sciatic nerve (3C, D) with similar percentages of atypical mitochondria as the mice treated with PFT-μ alone. No differences in mitochondrial size were measured when comparing the DRG and sciatic nerves of mice that received PFT-μ alone or PFT-μ + paclitaxel, 2E and 3E. Preliminary analysis of mitochondrial abnormalities did not show differences between vehicle alone (2a, 3A) or PFT- μ alone (2B, 3B) treated mice, therefore PFT- μ alone treated mice were used as baseline. The percentage of atypical mitochondria in samples from mice treated with PFT- μ alone was consistent with the percentage reported under baseline conditions in previously

published studies [54].” An image of mitochondrial morphology of a vehicle alone animal can be observed in Figure 2 and 3. These data demonstrate that PFT-μ efficiently preserves mitochondrial morphological integrity of peripheral sensory neurons in paclitaxel-treated mice.

The impact of PFT-μ on paclitaxel-induced IENF loss

We analyzed the potential protective effect of PFT-μ on loss of IENFs in the hind paws of paclitaxel-treated mice. The density of nerve fibers entering the epidermis was significantly reduced in the paclitaxel-treated mice (Figure 4B) when compared to PFT-μ + vehicle-treated mice (Figure 4A). Notably, PFT-μ administration significantly reduced paclitaxel-induced IENF retraction, Figure 4c and 4d. No differences in IENF levels were observed when comparing the PFT-μ and the PFT-μ + paclitaxel treated mice, Figure 4D. These data demonstrate that PFT-μ administration protects completely against paclitaxel-induced IENFs loss.

Potential effects of PFT on Tumor cell viability

One of the potential risks of treating CIPN with PFT-μ may be that the drug may also protect against paclitaxel-induced tumor cell death by preventing accumulation of p53 at the mitochondria. Therefore, we assessed the effect of PFT-μ on ovarian tumor cells treated *in vitro* with paclitaxel. Human ovarian ($p53^+$) tumor cells (HeyA8) were incubated with paclitaxel alone or in combination with PFT-μ. Tumor cell survival was measured by an enzymatic assay. Culturing HeyA8 cells with paclitaxel alone led to a reduction in tumor cell survival of more than 50% (Figure 5). Addition of PFT-μ to the cultures of tumor cells and paclitaxel further reduced ovarian tumor cell survival in comparison to paclitaxel alone (stripped bars). Additionally, PFT-μ (20 μM) alone also decreased tumor cell survival (solid bars). These data provide evidence that PFT-μ does not inhibit but even enhances the anti-tumor effect of paclitaxel.

The effect of PFT-μ administration on cisplatin-induced neuropathy

Next, we investigated if PFT-μ also prevented cisplatin-induced neuropathy. Mice were treated with cisplatin (2.3 mg/kg) alone or in combination with PFT-μ and mechanical allodynia was measured. In mice treated with cisplatin alone decreased paw-withdrawal thresholds were measured following cisplatin administration at weeks 3, 5, and 7 (Figure 6). Systemic PFT-μ administration completely prevented cisplatin-induced changes in paw-withdrawal threshold and thereby cisplatin-induced mechanical allodynia.

Discussion

We have identified the small molecule inhibitor PFT-μ as an efficacious agent to prevent chemotherapy-induced peripheral neuropathy (CIPN) in mice treated with paclitaxel or cisplatin. Moreover, PFT-μ completely prevented paclitaxel-induced changes in mitochondrial morphology in peripheral sensory neurons. Furthermore, PFT-μ administration protected against the loss of IENF in mice treated with paclitaxel. From these data we can conclude that PFT-μ prevents CIPN in mice treated with paclitaxel or cisplatin.

Notably, *in vitro*, PFT-μ does not inhibit but rather enhances the anti-tumor effects of paclitaxel.

Paclitaxel and oxaliplatin treatment have previously been shown to induce mitochondrial swelling and vacuolization in peripheral nerves[10; 52; 53]. *Ex vivo* analysis of peripheral sensory neurons demonstrated that chemotherapy-induced mitochondrial swelling resulted in decreased cellular respiration and ATP production [54]. Furthermore altered mitochondrial function was linked to increased spontaneous discharge of sensory neurons. This spontaneous neuronal discharge has been suggested to underlie neuropathic pain [50–52]. Loss of IENFs is associated with chemotherapy treatment as measured by a decrease in the density of nerve fibers entering into the epidermis [4; 5; 34; 38]. Mechanisms of how chemotherapy treatment leads to IENF retraction are less well understood. Some authors have proposed that IENF retraction is due to decreased mitochondrial transport in peripheral sensory axons [3; 54]. Others have suggested that the energy rich and high mitochondrial density specifically in nerve terminals renders these sites most sensitive to chemotherapy leading to retraction of the nerve ending [22]. The retraction of nerve fibers may leave the remaining nerve fibers highly sensitized and primed for spontaneous discharge. Here we report that PFT-μ administration preserves both mitochondrial morphology as well as IENF density following paclitaxel treatment. We hypothesize that PFT-μ is preventing p53-mediated mitochondrial disruption in peripheral sensory neurons. This prevention of mitochondrial disruption prevents further downstream consequences such as neuronal sensitization and IENF retraction.

When investigating potential therapeutics for CIPN, it is important to determine if the therapeutic candidates interfere with tumor cell-killing of chemotherapy. When we tested PFT-μ *in vitro* for its capacity to regulate tumor cell growth, we observed that PFT-μ does not impair, but actually enhances paclitaxel-induced tumor cell death. This observation is in line with other recent publications investigating PFT-μ as a potential anti-cancer agent. These studies showed that PFT-μ administration to human tumor cells in combination with other anti-tumor therapies (hyperthermia and chemotherapy) enhanced tumor cell death [15; 20; 24; 36]. Our work is the first to demonstrate that PFT-μ enhances paclitaxel-tumor cell death of HeyA8 human tumor cells. Additionally, we are the first to have identified PFT-μ as a potential efficacious therapeutic for CIPN. These results further highlight the importance of PFT-μ and its potential therapeutic benefit for CIPN and tumor growth.

The tumor suppressor gene p53 is upregulated following cellular stress signaling including DNA damage, hypoxia, and chemotherapy treatment [30; 42]. Mitochondrial accumulation of p53 regulates numerous cellular processes including apoptosis and autophagy. PFT-μ was discovered for its ability to prevent p53 accumulation at the mitochondria without impacting transcriptional activity of p53 [39]. In these initial drug screens Strom et al. investigated the protective capabilities of PFT-μ for radiation toxicity in mice [39] and showed that PFT-μ prevented weight loss and death of the animal associated with sub-lethal and lethal doses of whole body gamma radiation. PFT-μ has also been shown to prevent accumulation of p53 at mitochondria and to reduce mitochondrial oxidative stress in response to toxic chemical excitation of striatal neurons [8]. We previously investigated the protective effects of PFT-μ in a model of neonatal hypoxic-ischemic (HI) brain damage [30]. We showed that HI

increases p53 mitochondrial accumulation, reactive oxygen species (ROS) and cytosolic accumulation of pro-apoptotic proteins. These p53-mediated mitochondrial alterations led to neuronal apoptosis as measured by increased cytosolic cytochrome-c and active caspase-3 levels in association with white and grey matter damage in the brain. Systemic administration of PFT-μ prevented p53 mitochondrial accumulation, ROS production, release of pro-apoptotic proteins as well as HI-induced neuronal loss. In addition, PFT-μ administration prevented behavioral deficits such as motoric and cognitive function associated with HI-induced brain damage.

The data presented herein shows that paclitaxel administration leads to altered mitochondrial morphology and that this is prevented by co-administration of PFT-μ. Furthermore, this protection of mitochondria corresponds with prevention of chemotherapy-induced mechanical allodynia as well as IENF retraction. As a result, we have identified a means to prevent adverse behavioral and neuronal side-effects of chemotherapy. However, at present, we do not know whether PFT-μ exerts this protective effect by preventing p53 accumulation at the mitochondria as has been shown in our experiments focusing on neuronal protection by PFT-μ after hypoxic-ischemic brain damage.

It is surprising that PFT-μ protects neurons against chemotherapy-induced mitochondrial damage while at the same time enhancing tumor cell death. One reason for this dichotomy might be explained by studies which identified that PFT-μ can influence tumor cell death via interfering with the association between lysosome-associated membrane protein 2 (LAMP2) and HSP70 leading to accumulation of the waste recognizing molecule p62 which is involved in autophagia and tumor cell death [19]. The enhanced tumor cell death in response to PFT-μ was independent of p53 as PFT-μ- also induced tumor cell death in p53-null tumor cell lines. These data demonstrate that PFT-μ can exhibit tumor cell killing effects independent of p53. However, it should be noted that the effect of PFT-μ on p62 was observed in highly dividing (p53-) tumor cell lines. When Leu et al. investigated effects of PFT-μ on non-transformed fibroblasts they measured only limited effects on cell viability with no mention of increases in autophagy proteins. These data in combination with our study suggest that PFT-μ may have differential effects depending on either cell type or proliferative state of the cell [19]. We hypothesize that in a non-dividing cell such as a peripheral neuron, PFT-μ is predominantly interfering with chemotherapy-induced p53 mitochondrial accumulation thereby preventing sensitization and symptoms of CIPN. In tumor cells which are known to have increased levels of HSP70 [25], PFT-μ may primarily prevent HSP70 interaction with LAMP2 leading to accumulation of p62 proteins, dysregulation of autophagy and enhanced tumor cell death. Further studies will be required to determine what the mechanism via which PFTm protects against paclitaxel-induced mechanical allodynia.

The symptoms of CIPN can severely interfere with effective cancer treatment but can also persist long into survivorship thereby reducing quality of life [45]. The staggering numbers of individuals affected by CIPN make the discovery of efficacious therapeutics for treatment of CIPN extremely important. The findings in this work demonstrate that PFT-μ is an attractive candidate drug for prevention of CIPN since it has the potential to prevent CIPN and to enhance tumor-cell killing.

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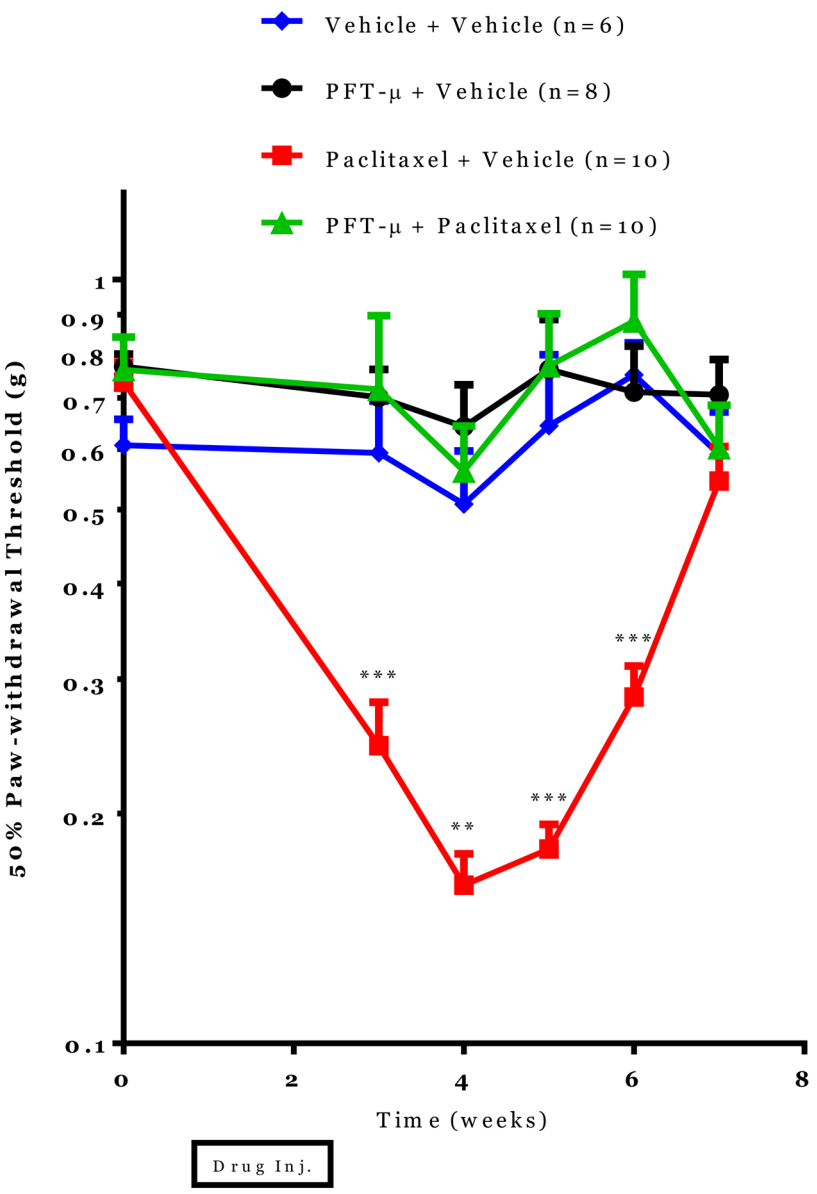

Figure 1. Effect of PFT-μ administration on paclitaxel-induced peripheral neuropathy

Mechanical allodynia was measured using von Frey hairs and the 50% paw withdrawal threshold was calculated by the up-down method. Statistical analysis using two-way repeated measures ANOVA revealed a significant group effect (p=0.0001), time effect (p=0.0006) and a time by group interaction (p=0.0006). Bonferroni post-hoc analysis was used to determine differences between groups at specified time points. *p<0.05, **p<0.01, ***p<0.001 between Paclitaxel+Vehicle and Paclitaxel + PFT-μ. n=6–10/group.

Figure 2A-D

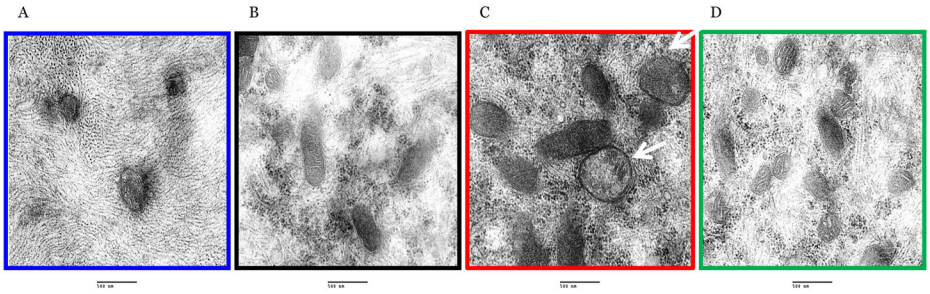

Figure 2a

Figure 2E

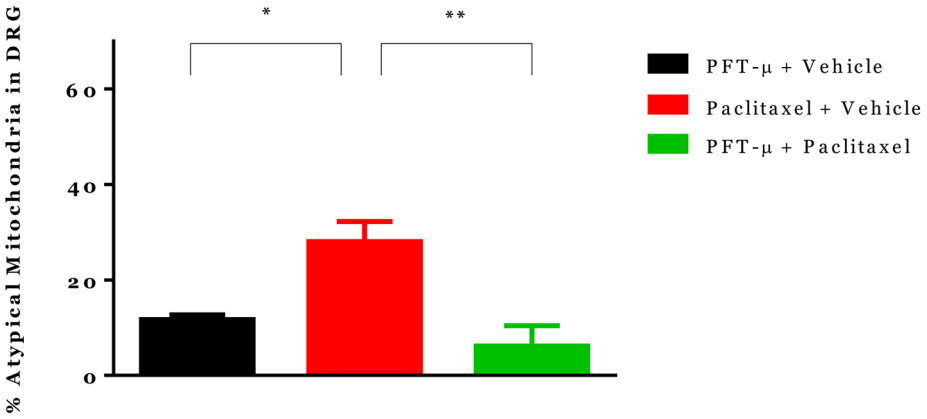

Figure 2b

Figure 2. The effect of PFT-μ administration on altered mitochondria morphology in peripheral sensory neuron cell bodies

Representative Electron Microscopy Images from Mitochondria in DRG from Vehicle alone (A) PFT-μ+Vehicle-Treated mice (B), from Paclitaxel-Treated mice (C); and from Paclitaxel +PFT-μ–Treated mice (D). DRG tissues were harvested at week 5 at the moment that paclitaxel-treated mice showed mechanical allodynia. Percentage of atypical mitochondria/ mouse (E). n=3 mice/treatment 10–25 mitochondria scored/mouse. Magnification 50,000 x. Statistical analysis using one-way ANOVA revealed a significant difference between groups ($p<0.01$). Bonferroni post-hoc analysis was used to determine differences between groups. *$p<0.05$, **$p<0.01$.

Figure 3A-D

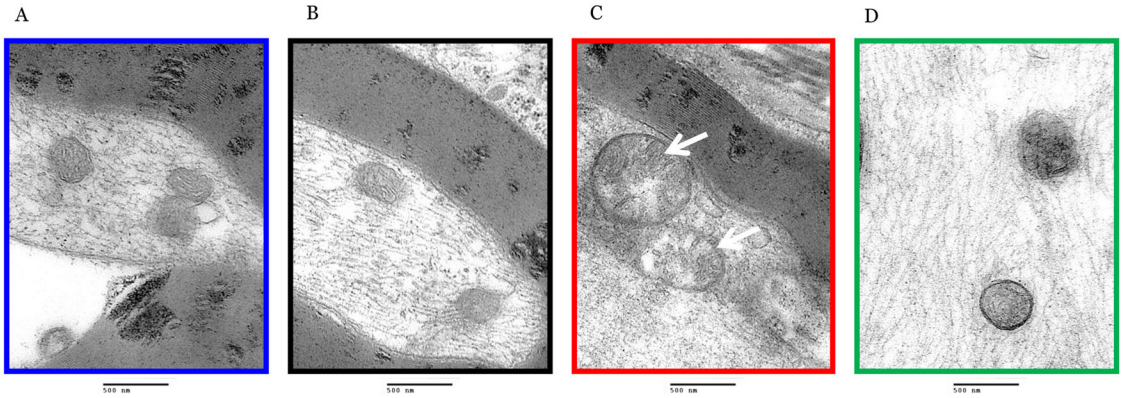

Figure 3a

Figure 3E

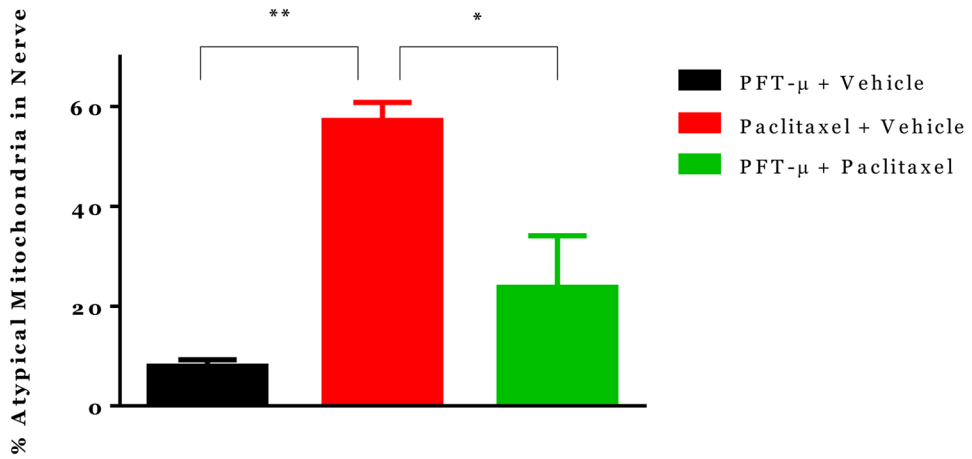

Figure 3b

Figure 3. The effect of PFT-μ administration on altered mitochondria morphology in peripheral sensory neuron axons

Representative Electron Microscopy Images from Mitochondria in peripheral sensory neuron axons (sciatic nerve) from Vehicle alone (A) PFT-μ+Vehicle-Treated mice (B), from Paclitaxel-Treated mice (C); and from Paclitaxel+PFT-μ–Treated mice (D). DRG tissues were harvested at week 5 at the moment that paclitaxel-treated mice showed mechanical allodynia. Percentage of atypical mitochondria/mouse (E). n=3 mice/treatment 7–18 mitochondria scored/mouse. Magnification 50,000x. Statistical analysis using one-way ANOVA revealed a significant difference between groups ($p<0.01$). Bonferroni post-hoc analysis was used to determine differences between groups. *$p<0.05$, **$p<0.01$.

Figure 4A-C

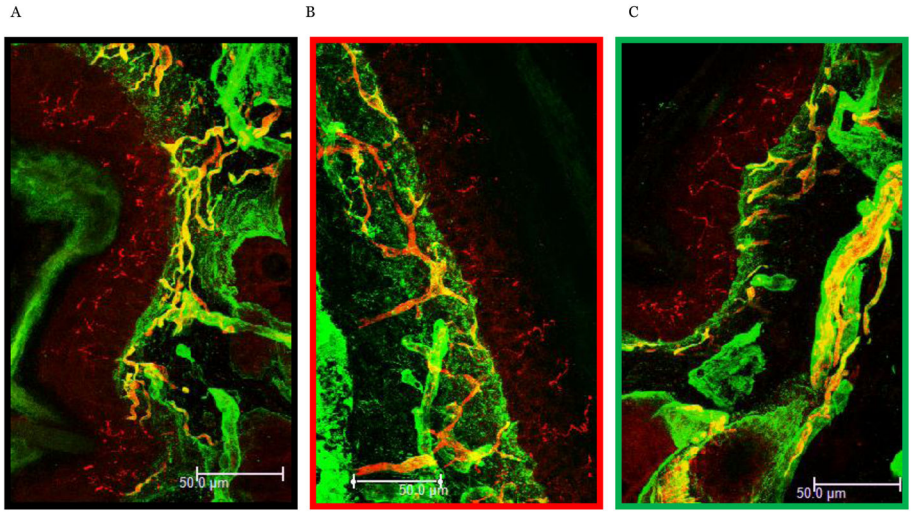

Figure 4a

Figure 4D

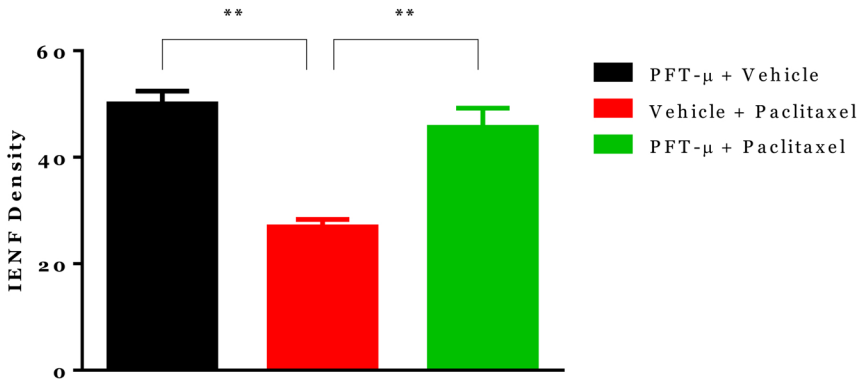

Figure 4b

Figure 4. The impact of PFT-µ on paclitaxel-induced IENF retraction

Paw biopsies were obtained from the hind paws of mice in Week 5 (when mechanical allodynia and mitochondria alterations were measured). Paws were fixed and stained with antibodies for intra-epidermal nerve fibers (PGP9.5; red) and collagen (green). To control for a-specific staining, paws (from each treatment group) were stained with secondary antibodies alone, which never resulted in positive staining. Representative images PFT-µ +Vehicle-Treated mice (A), from Paclitaxel-Treated mice (B); and from Paclitaxel+PFT-µ–Treated mice (C). Arrows denote intraepidermal nerve fibers (show in red) crossing the basement membrane (shown in green). Quantification of all paws obtained (n=3–4 mice/

group) shown in (D). IENF density = number of nerve fibers crossing the basement membrane/length of the basement member (mm). **p<0.01. Magnification 40x.

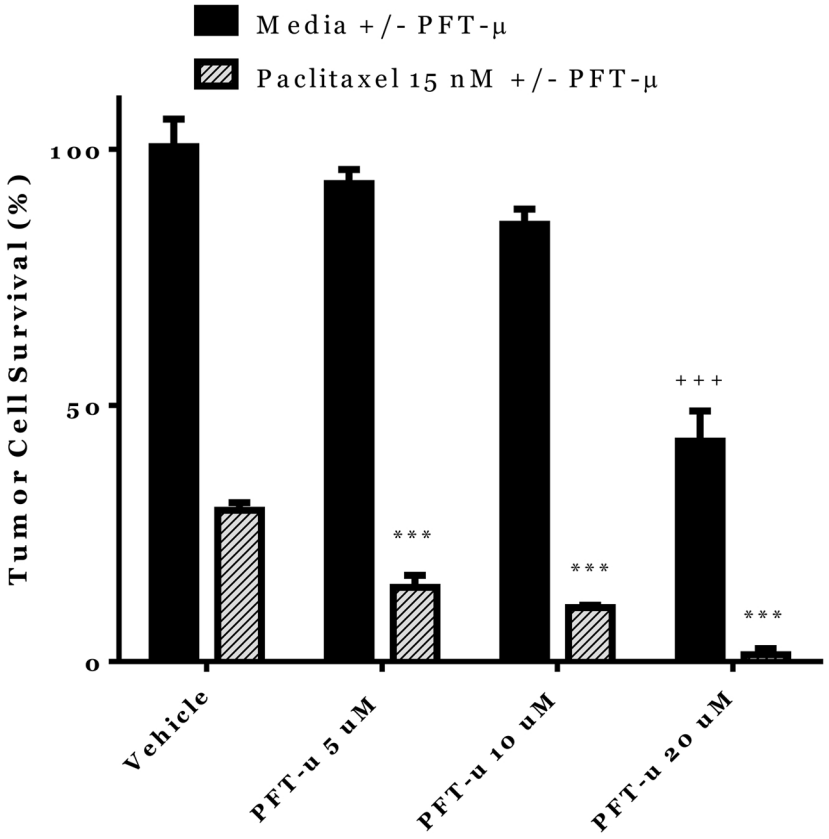

Figure 5. Potential effects of PFT on Tumor cell viability

Tumor cell (HeyA8 cells) survival as measured by MTT assay in the presence and absence of PFT-μ and paclitaxel (72 hrs.). Data were analyzed by two-way ANOVA. ***p<0.001 between Paclitaxel alone (Vehicle) and Paclitaxel+PFT-μ. +++ p<0.001 between PFT-μ alone (Vehicle) and PFT-μ 20 μM. n=4–12/group.

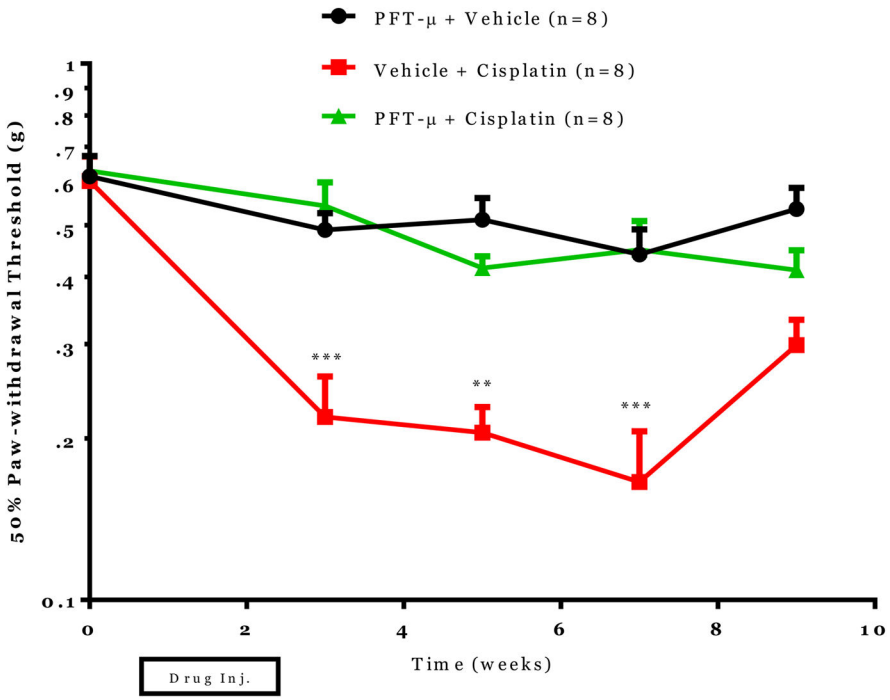

Figure 6. The effect of PFT-μ administration on cisplatin-induced neuropathy

Mechanical allodynia was measured using von Frey hairs and the 50% paw withdrawal threshold was calculated by the up-down method. Statistical analysis using two-way repeated measures ANOVA revealed a significant group effect (p=0.0001), time effect (p=0.0001) and a time by group interaction (p=0.0053). Bonferroni post-hoc analysis was used to determine differences between groups at specified time points. *p<0.05, **p<0.01, ***p<0.001 between Cisplatin+Vehicle and Cisplatin + PFT-μ. n=8/group.