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Mitochondrial trafficking as a protective mechanism against chemotherapy drug-induced peripheral neuropathy: Identifying the key site of action

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ABSTRACT

Aims: Chemotherapy induced peripheral neuropathy (CIPN) is a common side effect seen in patients who have undergone most chemotherapy treatments to which there are currently no treatment methods. CIPN has been shown to cause axonal degeneration leading to Peripheral Neuropathy (PN), which can lead to major dosage reduction and may prevent further chemotherapy treatment due to oftentimes debilitating pain. Previously, we have determined the site-specific action of Paclitaxel (PTX), a microtubule targeting agent, as well as the neuroprotective effect of Fluocinolone Acetonide (FA) against Paclitaxel Induced Peripheral Neuropathy (PIPN).
Main methods: Mitochondrial trafficking analysis was determined for all sample sets, wherein FA showed enhanced anterograde (axonal) mitochondrial trafficking leading to neuroprotective effects for all samples.
Key findings: Using this system, we demonstrate that PTX, Monomethyl auristatin E (MMAE), and Vincristine (VCR), are toxic at clinically prescribed levels when treated focally to axons. However, Cisplatin (CDDP) was determined to have a higher toxicity when treated to cell bodies. Although having different targeting mechanisms, the administration of FA was determined to have a significant neuroprotective effect for against all chemotherapy drugs tested.
Significance: This study identifies key insights regarding site of action and neuroprotective strategies to further development as potential therapeutics against CIPN. FA was treated alongside each chemotherapy drug to identify the neuroprotective effect against CIPN, where FA was found to be neuroprotective for all drugs tested. This study found that treatment with FA led to an enhancement in the anterograde movement of mitochondria based on fluorescent imaging.

1. Introduction

Chemotherapy Induced Peripheral Neuropathy (CIPN) is a debilitating disorder that affects >30–40 % of individuals treated with chemotherapy drugs [42]. CIPN negatively affects the peripheral nervous system, degrading sensory, motor, and autonomic neurons, causing patients to feel major discomfort and pain starting at the end of extremities [3]. Patients diagnosed with Peripheral Neuropathy (PN) experience tingling, sensory loss, and burning at the site of injury [29]. PN causes pathological changes coined as “Dying Back Neuropathies” where the distal axons are targeted and degrade towards the proximal cell bodies [52]. CIPN is a common side effect of anti-cancer drug treatments which can lead to inflammation, pain, and dosage reductions for patients undergoing chemotherapy treatment, to which there are currently no effective treatments for PN, other than symptomatic pain management [38].

Commonly used drugs for pain management and treatment of PN have a direct effect on the global neuronal environment in the nervous system [15]. PN pain management drugs such as duloxetine and gabapentin have an unfortunate side effect of being a Serotonin and norepinephrine reuptake inhibitors (SSNRI), which are primarily used to treat disorders such as depression and anxiety [8,23]. Such drugs primarily effect the inhibition of neurotransmitters, serotonin, and norepinephrine with a secondary effect on pain management for PN [20]. Side-effects and complications of using these types of drugs have significant repercussions on patients' quality of life and have the potential to cause discomfort, nausea, cyclothymia, and withdrawals among other common side effects [51]. Current drugs used for

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neuropathic pain management are not best suited to aid patients in treatment. Therefore, focal neuroprotection would be a better approach to determine specific treatment methods while avoiding side effects of conventional pain-relieving agents against CIPN.

In this study, we examined axonal susceptibility to degradation induced by four commonly used anti-cancer drugs: Cisplatin (CDDP), Monomethyl auristatin E (MMAE), Vincristine (VCR), Paclitaxel (PTX). The site of action for PTX has previously been determined and has been used as a reference model for the remaining drugs [52]. VCR and MMAE are common anti-cancer drugs which are known to target microtubule structure, similar to PTX [22]. For this reason, a comparison of the site of action for PTX, VCR and MMAE is necessary to further understand the fundamental mechanism of action for these drugs. CDDP likewise, is a common anti-cancer drug, however, it targets DNA replication, preventing tumor growth. CDDP was initially chosen due to its prevalence in chemotherapy treatment, as well as to use as a comparison drug with a different targeting mechanism. The site-specific mechanism of action for each of these drugs was determined in order to understand the focal susceptibility in vitro.

PTX is a well-known chemotherapy drug for breast, lung, and ovarian cancer [50]. It directly targets the mitotic spindle during mitosis, which leads to disruption of cell division and can help stop tumor development and cancer cell growth [50]. PTX is also a known drug that induces CIPN in patients, leading to heavy dosage reductions and treatment alterations [32]. PTX has been shown to induce dying back neuropathy, where distal axons are more susceptible to degradation by PTX [52]. Recently, Fluocinolone Acetonide (FA), has been shown to be neuroprotective against Paclitaxel induced peripheral neuropathy (PIPN) [13]. The use of FA can protect sensory neurons from PIPN, without reducing the anticancer effects of PTX, making it a valued, potential neuroprotective agent against CIPN. Additionally, MMAE is a well-known and commonly used Chemotherapy drug similar to PTX used to treat relapsed Hodgkin's lymphoma and anaplastic large cell lymphoma [18]. MMAE in itself is not approved directly for cancer treatment, however in conjunction with an antibody-drug conjugate (ADC), MMAE is linked to a monoclonal antibody which allows MMAE to specifically target a cancer cell [19]. Antibody drug conjugates (ADC) are used to focally target the cancerous cells through administration of the drugs directly at the cancerous tumor [5]. By using ADC, extremely toxic drugs such as MMAE are able to be administered without having as significant of an effect on the global cellular environment [9]. Similar to PTX, MMAE is a microtubule-impacting agent which in clinical settings, leads to CIPN complications in patients [9]. The neurotoxicity of MMAE is not fully known, however MMAE tends to cause peripheral sensory, and motor neuropathy leading to impactful dosage reductions, and occasionally treatment discontinuation. Similarly, VCR is a known Vinca Alkaloid drug used for cancer treatment. VCR targets microtubule polymerization to prevent mitosis associated with cancer cells similar to PTX and MMAE [6]. VCR is an FDA approved anti-cancer drug used for patients who suffer from Ewing sarcoma, gestational trophoblastic tumors, multiple myeloma, ovarian cancer, primary CNS lymphoma, small cell lung cancer, and advanced thymoma [6,26]. VCR is known to cause peripheral sensory and motor neuropathy, which leads to dosage modification and loss of effectiveness during treatments [47,48]. VCR is a dose dependent drug where PN was shown in patients when administered 4–10 mg [48]. Due to the widespread use of VCR as a treatment method, it is imperative to understand the neurotoxicity mechanisms and identify treatment methods to prevent CIPN onset. Due to the similarities between MMAE, VCR and PTX, it is assumed that distal axons would be more susceptible to degradation as seen with PTX and herein we tested this hypothesis to study the site of action. On the other hand, CDDP is a platinum based antineoplastic drug used for chemotherapy treatment against testicular, ovarian, bladder, head and neck, lung, and cervical cancer [43]. CDDP targets DNA replication by binding to DNA, directly affecting replication and transcription [43]. CDDP is referenced in the world health organization's list of essential medicines, targeting many types of tumors [34]. CDDP has been seen to cause axonal degeneration, which is the primary dose limiting factor for CIPN [2]. Specific mechanisms of toxicity from CDDP are not yet known, however it is speculated that, peripheral sensory neurons, changes in cell signaling cascades, changes to calcium homeostasis and signaling, oxidative stress, mitochondrial dysfunction and induction of apoptosis as a result of DNA platination, are all potential mechanisms behind cisplatin-induced peripheral neuropathies [30].

A dosage response for each drug was identified to choose proper concentration levels for CIPN analysis. To better understand the specific site of action of each drug, a differentiation of cell bodies to axons was created via a PDMS based microfluidic chamber system [52]. Previous studies have not yet determined if the cell bodies or axons are the site-specific targets for the drugs [4]. Using the compartmentalized microfluidic cell culture platform, we demonstrate that axons are more susceptible to the toxicity induced by the chemotherapy drugs which target micro-tubules (PTX, MMAE, VCR), whereas cell bodies are more susceptible for DNA targeting samples (CDDP) in vitro. Using this information, more effective treatment methods can be developed to specifically target the most vulnerable region in CIPN patients.

Previously, it has been shown that the FDA approved drug, FA, is neuroprotective against PTX [13]. In a similar manner, we have identified the neuroprotective effect of FA on the FDA approved chemotherapy drugs. The findings of this study are important for identification of site of action for degradation susceptibility and FA-induced neuroprotection. In this study, we determine the role of Fluocinolone Acetonide (FA) in mitochondrial enhancement as a neuroprotective mechanism against common CIPN. The observation of focal susceptibility is an important factor in creating effective treatment methods for patients experiencing CIPN. Treatments may vary depending on the susceptibility whether at the site of injury (axon) or at the cell body imbedded in the spinal cord. This distinction helps understand the fundamental mechanism behind CIPN and how to develop effective strategies to combat axonal degeneration in patients who suffer from CIPN.

Independent to site of susceptibility, we determine mitochondrial trafficking response to treatment with chemotherapy drugs [31]. It is speculated that CIPN mechanism is induced through mitochondrial dysfunction caused by drug administration [46]. In this study, we determine the effect of each stated drug on mitochondrial trafficking and determine its correlation with CIPN. As mitochondrial motility is an essential subcellular mechanism for axon growth and general health, it is essential to understand how CIPN drugs effect mitochondrial trafficking [14,41]. Using this information, new therapeutic strategies can be developed to enhance mitochondrial trafficking in CIPN patients.

2. Materials and methods

2.1. Preparation of compartmentalized chamber

The master mold used to create the microchannels for the compartmentalized device was produced using a photolithographic method. Standard silicon wafers were coated with SU-82002 and spun and soft baked according to the manufacturer's specifications, to result in a thickness of 2.5–3 μm of the resist. The microchannels with the following dimensions: width of 10 μm, length of 500 μm, and depth of 3 μm, were cured by UV light exposure using high resolution DPI transparency. The same process was repeated using SU-83050 to help outline the chambers with a width of 3 mm, and length of 13 mm. Sylgard 184 polydimethylsiloxane (PDMS) (Dow chemical company USA) was used to conduct standard soft lithography, where it was poured onto the master micro-mold developed in house followed by the removal of bubbles by a desiccator (SP Scienceware, USA) and was allowed to cure overnight at 80 °C. PDMS was removed from the master mold where two adjacent holes were punched using a 6 mm diameter biopsy punch (Robbins Instruments). The dual compartmentalized chambers were

then bonded to a thin glass slide (Fisher Scientific) using the Plasma Etch PE-75 Plasma Asher oxygen plasma device. After oxygen plasma bonding, the chamber devices were sterilized by autoclave before cell seating. Once sterilized, microchannels were cleaned using 98 % ethanol (Carolina Biological) and further diluted out using Distilled water. Chambers were coated with Poly-D-Lysine (PDL) (Sigma-Aldrich) and Laminin (Corning) and left overnight at 4 °C. Chambers were then washed thoroughly using media to prepare for cell seating. Chambers were then placed into a sterilized primary cell incubator, Binder C-150 UL Incubator.

2.2. Dorsal root ganglion culture

All experiments related to animals were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). Dorsal Root Ganglion (DRG) neurons were isolated from an E-15 Sprague Dawley Rat. The DRG explants were then enzymatically dissociated with 0.25 % Trypsin in L15 medium (Sigma Aldrich) and suspended in media. DRGs were maintained in Neurobasal medium containing 1 % fetal bovine serum (FBS), 20 % glucose, 1 % penicillin/streptomycin, B-27 supplement, 2 M L-glutamine, and 10 mg/mL glial derived nerve growth factor (GDNF) (Sigma Aldrich). Neurobasal media that contains 10 μM of Cytosine arabi-noside was added to the cultures, two days after seeding the cells, to help decrease the number of glial cells and other cell type contamination. To further limit evaporation, a small cotton ball soaked in sterile distilled water and 1.0 % Penicillin/Streptomycin was placed in the same petri dish as the chambers. DRG neurons were seated into the marked somal chamber of the devices and left to grow for 5–7 days to allow for axons to grow through the channels into the axonal side at an adequate length.

2.3. Evaluation of FA interference on chemotherapeutic activity

Human SKOV-3 Ovarian cancer cells were seated in a 96 well culture chamber using 1×10^4 cells in 100 μL of media. SKOV-3 cells were incubated 24 h prior to drug treatment. Cancer cells were then treated using CDDP 10 μM; MMAE 1 μM; VCR 5 μM; PTX 5 μM respectively with varying concentrations of FA 10 nM–100 nM. Cells were stained with Hoechst 33342 stain (Invitrogen) 48 h after drug treatment in order to measure cell viability. Fluorescent images were captured using a Leica DMI8 thunder Microscope. Cell count was determined using Fiji-ImageJ, where fluorescent images were processed by, first, making the image 16 bit, then changing the threshold so that only the cells were identified. After threshold alteration, the image was converted to binary and made into a mask, where accurate cell count was measured using the analyze tool.

2.4. Preparation of cancer drugs

Stock solutions for each anti-cancer drug were prepared using the necessary concentration needed for each experimental application and data concentration. A stock solution of 1 mM was prepared, where MMAE (Selleck chemicals) and VCR (Selleck chemicals) were dissolved in DMSO while CDDP (Sigma Aldrich) was dissolved in NaCl, and PTX (Selleck chemicals) was dissolved in ethanol, where they were then stored at −20 °C. The concentrations used were based off the Clinical concentration published [28], which demonstrate neurotoxicity in vitro models to help achieve similar conditions as those in real world application. Different concentrations for each of the drugs was made in culture medium using the stock solution for the dosage response, cell body and axonal treatment and stored in −4 °C. The drugs were administered on either the cell body or the axonal side for each experiment by removing approximately 75 % of the existing media and readministering with the drug in question. The cells were stained using calcein AM (ThermoFisher) dye for 30 min–1 h and imaged using a Leica DMi8 Thunder Fluorescence Microscope immediately after the drug was administered as well as 24 h after treatment. Mitoview Orange was obtained from Biotium, USA, and was used to stain the somal chamber containing cell bodies prior to drug treatment for 30 min–1 h. Mitochondria were imaged immediately after dilution of the dye using the Leica DMi8 Thunder Fluorescence and then again, 24 h after initial imaging.

2.5. Data analysis

Data was collected using Fiji-ImageJ to measure axon lengths in triplicate samples. Microsoft Office Excel 2022 was used to compile all measurement data, where average and standard deviation was calculated. Axon integrity was found by calculating percent difference compared to the average control (0 h) for each sample. Differences in percent change were used to analyze the effect of site-specific treatment. All control samples are characterized by untreated conditions in complete neurobasal media.

2.6. Statistical analysis

All data sets were presented as a mean ± standard deviation except where noted. Data sets were grouped as triplicate. The probability *(P*-value*)* between groups was analyzed by the two-tailed *t*-test provided in Microsoft Office Excel 2022 unless otherwise stated. P-value < 0.05 was considered statistically significant.

3. Results

3.1. Global response

Fig. 1 illustrates the fundamental experimental design used to perform the site of action analysis. A microfluidic compartmentalized cell culture device was used to selectively isolate the cell bodies and axons for focal administration of anti-cancer drugs. Aside from the microchannels, the chambers were open air and were subjected to 5.0 % Carbon Dioxide to maintain a high buffering capacity. The microchannels were able to hold approximately 250 μL of volume. Equal volume of media was placed into the chambers, making sure to keep hydrostatic pressure equal and prevent diffusion of media from one chamber to the next. After drug administration hydrostatic pressure was changed by adding an extra 50 μL of media to the untreated chamber to ensure the drugs would not diffuse to the adjacent (untreated) chamber. Axons traveled through the microchannels within 3–5 days and were monitored continuously until proper axon length could be determined.

To determine axonal susceptibility to CIPN, the dosage response of each chemotherapy drug was observed to understand the neurotoxicity on sensory DRG neurons. Different concentrations of each drug were analyzed based on previous literature and clinical data [10,33,39,40]. Global (cell body and axonal) response was imaged 24 h after drugs were added. Fiji-ImageJ was used to measure axon lengths for each corresponding drug and concentration where axon length was analyzed. In Fig. 2, we show the dosage response for each drug as well as each drug alongside FA and the control samples used were untreated DRG's that were under the same conditions and imaged at the same time as the other cells. Each drug induced a state of peripheral neuropathy on the sensory DRGs shown as a decrease in axon length. All drugs observed show that axon degeneration is dependent on dosage of each drug. Optimal concentrations of each drug were used for further analysis of axon susceptibility to CIPN.

DRG neurons were seated in a glass bottom 96 well cell culture system 24 h prior to drug administration. After drugs were added in different concentrations, axon lengths were measured using Fiji-ImageJ. Shown in Fig. 2 A-D, the axon lengths were measured in comparison to the control group. The neurotoxic effect of the chemotherapy drugs can be seen with in vitro conditions in Fig. 2. Using the appropriate toxicity response of each drug observed, FA was added in conjunction to the

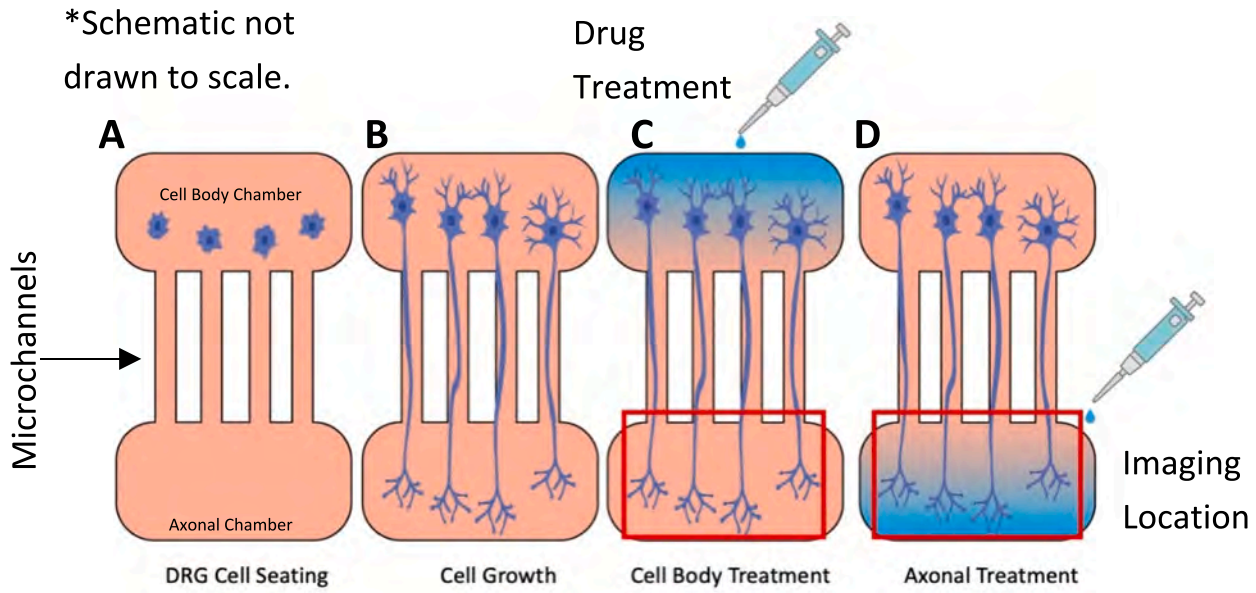

Fig. 1. 2D-Schematic of experimental methods. Panel A illustrates the cell seating of the isolated DRG's. Panel B illustrates the cell growth of the isolated DRG's after 3–5 days. Panel C illustrates the cell body treatment of the isolated DRG's with chemotherapy drugs. Panel D illustrates the Axonal treatment of the isolated DRG's with chemotherapy drugs. The red box represents the region of examination by fluorescent microscopy.

drugs, and was used to determine the neuroprotective effect on the global cell treatment. Axon length measurements were measured for each drug and corresponding FA treatment. The dosage response was used to identify the optimal concentration for each of the drugs, which is 10 μM (1.67 mg/kg) for CDDP and 10 μM/20 nM for CDDP/FA, 1 μM (0.1–3.2 mg/kg,) for MMAE and 1 μM/20 nM for MMAE/FA, 5 μM (0.013–0.047 mg/kg) for VCR and 5 μM/100 nM for VCR/FA, 5 μM (4.5–5.8 mg/kg) for PTX and 5 μM/10 nM for PTX/FA.

For CDDP 28.12 ± 37.28 μm was recorded, while FA treatment showed, CDDP/FA 217.66 ± 101.37 μm. For MMAE 20.47 ± 17.33 μm was recorded, while treatment showed, MMAE/FA 92.11 ± 21.71 μm. For VCR 47.42 ± 18.83 μm was recorded, while FA treatment showed, VCR/FA 39.39 ± 31.32 μm. Additionally, For PTX 102.85 ± 32.33 μm was recorded, while FA treatment showed, PTX/FA 198.82 ± 81.05 μm. Using the microfluidic cell culture platform, DRGs were seated in the cell body chamber where axons were allowed to grow through the microchannels, to the axonal chamber for 3–5 days. Cells were monitored every 24 h until axons could be seen microscopically in the axonal chamber. Using the compartmentalized model, the site of action of chemotherapy drugs was analyzed to further understand the mechanism of toxicity. Drug concentrations were applied to the cell body *or* the axonal chamber, where axon lengths were recorded as seen in Figs. 3 and 4. Imaging analysis was done at 0 and 24 h for each drug. Axon lengths were measured using Fiji-ImageJ to understand the axonal susceptibility of each drug. Fig. 3 represents drug treatment in the *axonal* chamber, showing 0 and 24 h after focal treatment. Fig. 4 represents drug treatment in the *cell body* chamber, where response was analyzed 0 and 24 h after focal treatment. In Fig. 3, axons are shown to be more susceptible to CIPN compared to cell body application in Fig. 4. The chemotherapy drugs used, targeted axons of DRGs, demonstrating axonal susceptibility in CIPN.

The dosage response data analyzed in Fig. 2 allows for a better understanding of the global cellular response to chemotherapy drugs and their concentrations used in this study: PTX 5 μM, MMAE 1 μM, CDDP 10 μM, and VCR 5 μM. Analysis of PN induced by the selected drugs was performed in the same conditions to reduce any external effects. A noticeable decrease in axonal length is a result of peripheral neuropathy and was identifiable by comparison of the axonal length pre and post drug treatment. Based on the graphical data, a toxicity response of each chemotherapy drug was shown. Optimal concentrations based on Fig. 2 were used for the remaining study. Clinical concentration of the chemotherapy drugs help highlights the concentrations used in the dosage graph to show each drugs neurotoxicity in comparison to real world application [4]. PTX was found to be administered at a dose of approximately 4.5–5.8 mg/kg, MMAE at a dose of 0.1–3.2 mg/kg, CDDP at a dose of 1.67 mg/kg, and VCR at a dose of 0.013–0.047 mg/kg. Shown by Fig. 2, FA had the ability to protect against degradation induced by CDDP, 55.21 %, MMAE, 32.62 %, VCR, 43.86 %, and PTX, 48.27 % compared to drug treatment without FA on average. These values identify the neuroprotective effect of FA on the four different CIPN drugs outlined. Although there are different mechanisms for the drugs used, FA was found to be neuroprotective in all cases, and even resulting in similar protection levels.

In order to identify FA as a neuroprotective drug against common anticancer drugs, the effect of FA on the anticancer ability of each drug was characterized. SKOV-3 Human Ovarian cancer cell lines were used to determine the effect of FA at different concentrations used for the dosage response in Fig. 2. Seen in Fig. 3, Hoechst stain was used to identify cell viability count after 48-h treatment. Based on the results, FA did not have any significant negative effect on the anticancer activity for each drug. Based on these results FA is shown to be an effective drug to test its neuroprotective ability on DRGs against common chemotherapy drugs. For CDDP cell viability of 0.1939 % was recorded, while FA treatment showed, CDDP/FA was 0.2080 %. For MMAE cell viability of 0.0291 % was recorded, while FA treatment showed, MMAE/FA was 0.0360 %. For VCR cell viability of 0.0510 % was recorded, while FA treatment showed, VCR/FA was 0.0441 %. Additionally, For PTX cell viability of 0.0085 % was recorded, while FA treatment showed, PTX/FA 0.0077 %. The maximum difference in cell viability seen by FA treatment was still seen to be <0.02 % indicating a non-significant change. These results identify that FA does not inhibit anticancer effect of the drugs used, therefore it does not prevent the activity of the drugs from killing cancer.

3.2. Site of action

Axonal susceptibility was analyzed using the compartmentalized platform where drugs were added to the cell body and axonal chamber with FA treatment and the control samples used were untreated DRG's that were under the same conditions and imaged at the same time as the

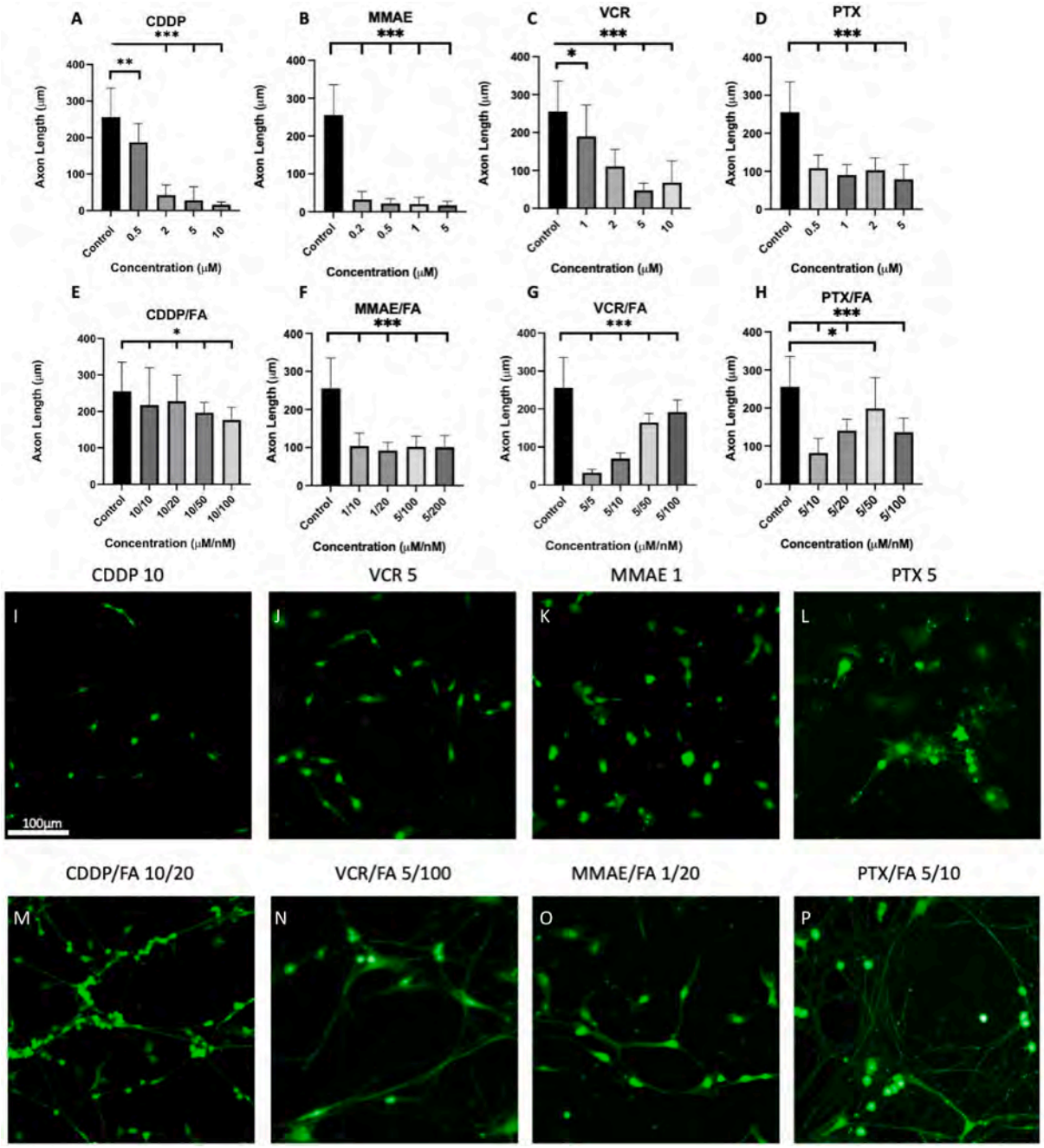

Fig. 2. Axonal response to local chemotherapy drug treatment. Axonal length measurement 24 h after administration of chemotherapy drugs (A-D). Axonal length measurement of drug concentration with increasing FA concentration (*E*-H). Corresponding global treatment and cellular response of chemotherapy drugs (I-L), and of chemotherapy drugs with FA (M-P). Optimal concentrations determined were, CDDP and CDDP-FA was imaged using 10 μM and 10 μM/20 nM. MMAE and MMAE-FA was imaged using 1 μM and 1 μM/20 nM. VCR and VCR-FA was imaged using 5 μM and 5 μM/100 nM. PTX and PTX-FA was imaged using 5 μM and 5 μM/10 nM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

other cells. Shown in Fig. 5 axon integrity was measured by comparison of axon length between 0- and 24-h timestamps for each sample. Percentage integrity was determined based on the length difference compared to the control. Data collected shows the axonal susceptibility compared to the cell body response. Fig. 5. Shows apparent axonal susceptibility with VCR and PTX, however as expected, CDDP is more susceptible on the cell body side due to its different targeting mechanism. MMAE is seen to have similar results between the cell body and axonal treatment samples, with FA having a slightly higher neuroprotective effect with axon treatment. We believe that the high toxicity of MMAE could be the reason why there is no specific site of action observed for MMAE. Based on Fig. 5. We have identified that FA is neuroprotective against both targeting mechanisms associated with CIPN. FA not only protected against PN for all drugs but even had some regeneration effect for both microtubule and DNA targeting mechanisms. Based on these findings we can assume that neuroprotection by FA can be attributed to enhanced mitochondrial trafficking.

3.3. Neuroprotection

Fig. 6 identifies the mitochondrial trafficking effect on axonal treated samples, with and without FA administration and the control samples used were untreated DRG cells were under the same conditions and imaged at the same time as the other cells. Fluorescent images of

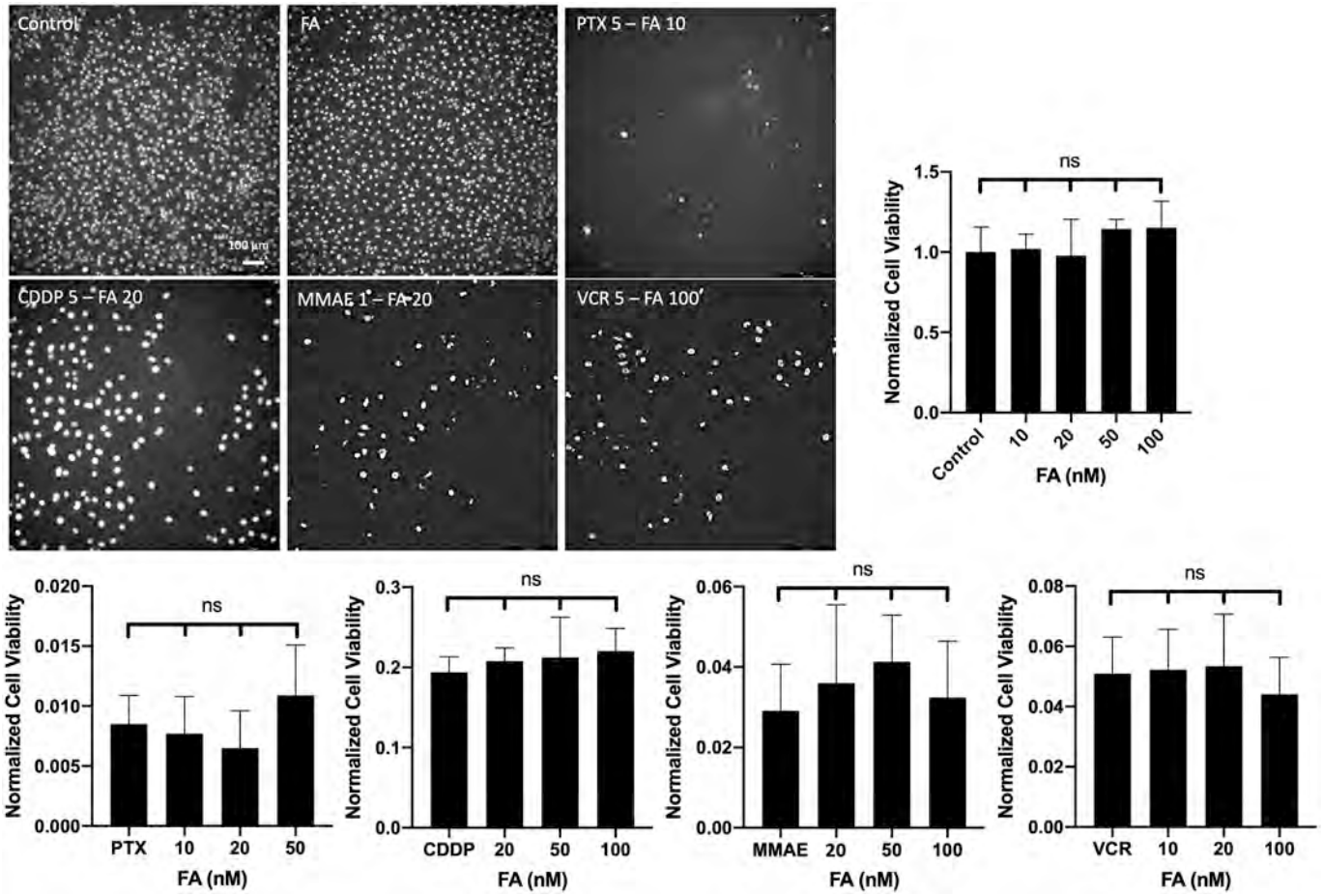

Fig. 3. Anticancer effect of CDDP, MMAE, VCR, and PTX with varying FA concentrations. Representative images of cell viability after 48 h using Hoechst stain (A). FA only treatment using, 10 nM, 20 nM, 50 nM, and 100 nM concentrations (B). PTX treated samples at 5 μM with varying concentrations of FA, 10 nM, 20 nM, and 50 nM (C). CDDP treated samples at 10 μM with varying concentrations of FA, 20 nM, 50 nM, and 100 nM (D). MMAE treated samples at 1 μM with varying concentrations of FA, 10 nM, 50 nM, and 100 nM (E). VCR treated samples at 5 μM with varying concentrations of FA, 10 nM, 20 nM, and 100 nM (F).

mitochondria were captured using Mito-Orange at 0 h and 24 h after drug treatment. The open-source Image J software was used to quantify mitochondria numbers along the microchannels. We used a size-based mask to identify the individual mitochondria (5–50 μm in size) typically observed in axons and to eliminate fluorescent debris along the micro-channel. Fig. 6. Shows clear mitochondrial inhibition due to CIPN degradation. Mitochondrial population decreased 73.45 ± 9.57 % for CDDP, 92.83 ± 6.25 % for MMAE, 45.58 ± 24.67 % for VCR, and 69.60 ± 12.85 % for PTX. For all drug samples, mitochondrial population was diminished significantly.

The treatment of FA alongside CDDP, MMAE, VCR, and PTX shows an increased mitochondrial number along the axons in comparison to the control sample. It is significantly noticeable in CDDP, where 83.45 ± 9.57 % increase is seen. This enhanced anterograde mitochondrial trafficking when treated with FA, resulted in axonal neuroprotection.

Using mitochondrial dyes, it is possible to see an increase in mitochondrial movement towards distal axons when focally treated. By comparison, it can be assumed that FA's effect on microtubules and mitochondria is a primary factor for neuroprotection against CIPN onset. Data provided shows that FA was neuroprotective against CDDP 39.5 % in axonal focally treated samples as seen by Fig. 5A. Initially it was assumed that CDDP would not have a negative effect on cells when focally treated on axons, however, it was clear to see CIPN induced from CDDP focally treated axons in vitro. Data provided showed that CDDP directly affects the distal axons of DRGs, and FA was found to be neuroprotective in axonal treated samples. This new finding is exciting to develop a better understanding of the mechanism of CIPN induced by CDDP as well as the neuroprotective mechanism of FA on CIPN.

Mitochondrial motility was characterized by performing timelapse imaging of fluorescently stained mitochondria over a 30-min time period with 1-min intervals. Displacement was measured using Fiji-ImageJ where mitochondria movement can be seen to decrease when treated with chemotherapy drugs. Fig. 7A shows FA's ability to increase mitochondrial displacement during the 30-min period compared to non-FA treated samples.

4. Discussion

Peripheral Neuropathy (PN) is a common and dose-limiting side effect frequently observed in patients undergoing chemotherapy treatment [16]. Previous studies have not identified the site of action or specific mechanism of the degenerative effect that takes place [27,49]. The in vitro microfluidic device used in this study allowed for demonstration of axonal and cell body susceptibility to CIPN and FA neuroprotection. This model enabled us to separately treat the cell body and axonal compartment for each drug sample and examine the degenerative result, which helped clarify important mechanisms behind axonal degeneration induced by CIPN [11].

.Although degeneration effects were seen using the global, cell body, and axonal models, based on the graphs in Fig. 5, it is clearly seen that axons are more susceptible to degradation in focal axonal treatment on average for MMAE, VCR, and PTX. However, CDDP was seen to cause higher levels of degradation with cell body exposure, as expected from our initial hypothesis as it is a known DNA replication disruptor [30].

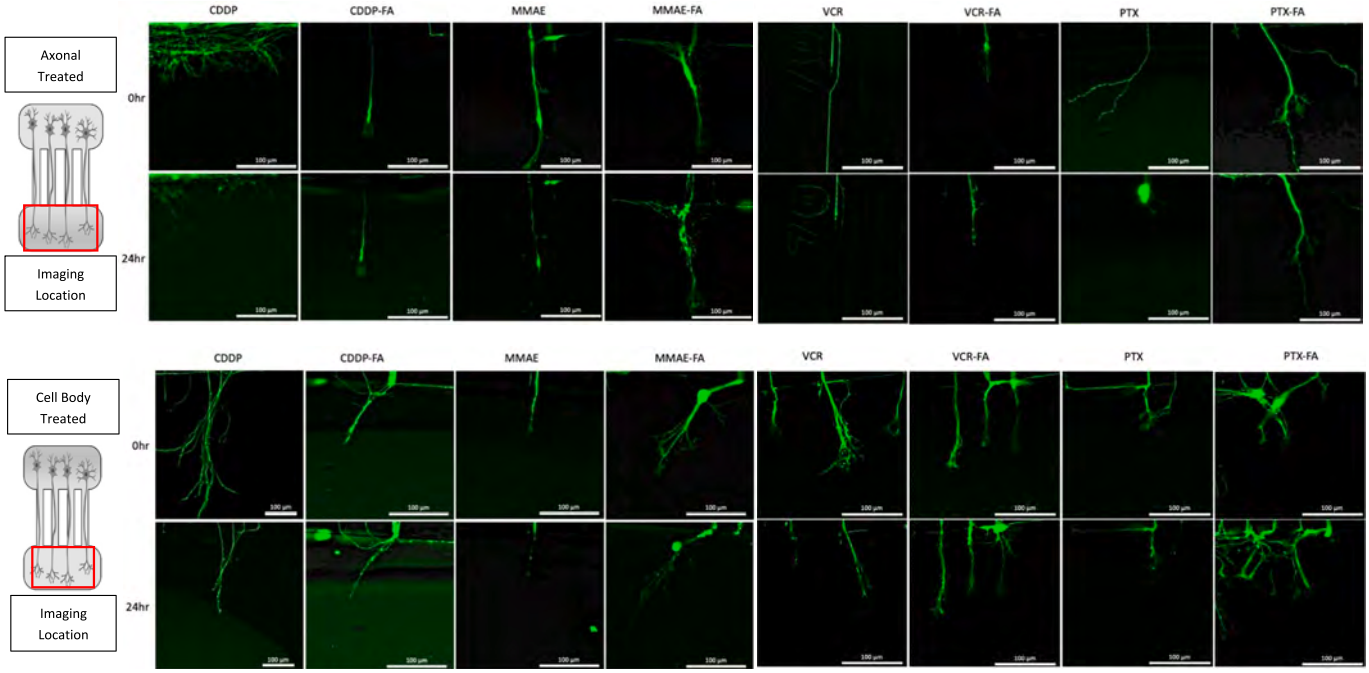

Fig. 4. Axonal response to focal Cell Body treatment of chemotherapy drugs. Fluorescent images at 0 h and 24 h at the same location for each sample. CDDP (A) and CDDP-FA (B) was imaged using 10 μM and 10 μM/20 nM. MMAE (C) and MMAE-FA (D) was imaged using 1 μM and 1 μM/20 nM. VCR (E) and VCR-FA (F) was imaged using 5 μM and 5 μM/100 nM. PTX (G) and PTX-FA (H) was imaged using 5 μM and 5 μM/10 nM. Axonal response to focal Axon treatment of chemotherapy drugs. Fluorescent images at 0 h (A-H) and 24 h (I-P) at the same location for each sample. CDDP (I) and CDDP-FA (J) was imaged using 10 μM and 10 μM/20 nM. MMAE (K) and MMAE-FA (L) was imaged using 1 μM and 1 μM/20 nM. VCR (M) and VCR-FA (N) was imaged using 5 μM and 5 μM/100 nM. PTX (O) and PTX-FA (P) was imaged using 5 μM and 5 μM/10 nM.

CDDP was the only platinum-based drug that primarily targeted the DNA replication while the other chemotherapy drugs targeted microtubule structure. Since the vast majority of DNA is located in the cell body, CDDP was expected to have significantly higher degeneration effect when exposed to the cell body. However, this poses the question, if CDDP primary targets the cell body, why does it cause peripheral neuropathy rather than acute cell death? Likewise, why does CDDP also result in degeneration when cell bodies are not exposed?

Previously, it had been determined that FA was found to be neuroprotective against PTX induced CIPN [45]. Based on these findings, FA was treated alongside MMAE, VCR, and CDDP to determine the neuroprotective mechanism against the drugs using microfluidic chambers. Similar to PTX; MMAE and VCR directly target the microtubules of the cell, leading to inevitable cell death. Herein, we focally administered FA to each drug treated sample to gain a better understanding of its neuroprotective mechanism against CIPN. Optimal concentrations were used based on the global dosage response data provided in Fig. 2. Based on the data collected, our results have shown that FA is neuroprotective against CIPN for each drug analyzed, leading us to assume that FA targets a mechanism involved with microtubule formation and organelle transport. FA was also found to be neuroprotective against CDDP which is known to affect DNA replication. Focal administration of FA treatment with CDDP shows neuroprotection against CIPN in vitro. Recently, we discovered FA's role in mitochondrial transport and its effect on retrograde and anterograde mitochondrial trafficking [45]. Previous studies have identified mitochondrial dysfunction associated with PIPN, however they do not discuss the mitochondrial effect of other common CIPN drugs [21]. Studies have shown that CDDP directly affects the DNA of neuronal cells, however, we have shown neuroprotective effect of FA alongside CDDP when administered focally on distal axons [12]. The neuroprotective effect of FA shows that CDDP may not affect only DNA in the nucleus of the cell, but also the DNA of mitochondria in the axon [36]. Based on this, it is believed that FA enlists mitochondrial movement, and recruitment at the site of injury [45]. FA is likewise known to suppress membrane permeability due to its nature as a corticosteroid [24]. The ability to suppress membrane permeability is a potential reason as to why CDDP is unable to effect mitochondria as compared to without FA treatment. By blocking CDDP from permeating mitochondrial membrane, FA is able to protect axonal mitochondria from damage.

Mitochondrial motility characterized by Fig. 7 shows an increase in mitochondrial movement when treated with FA compared to non-FA treated samples. Although Fig. 7 shows minor increases in movement, this can be attributed to experimental conditions associated with timelapse imaging. Significant changes in axonal structure, mitochondrial movement, and dye degradation make timelapse imaging difficult to produce accurate results. For this reason, the immediate effect of FA on chemotherapy treated samples was determined by treating cells 1 h prior to imaging. Due to the time-dependent nature of the chemotherapy drugs tested, significant axonal degeneration, and mitochondrial dysfunction may not be able to be shown by this result. However, the immediate change in mitochondrial trafficking helps give a better representation of motility associated with drug treatment. Although the results shown do not show drastic differences, Fig. 7. Shows an accurate representation of the immediate effect on mitochondrial trafficking by chemotherapy drugs and FA treatment. Further velocity characterization is needed to identify the full effect of chemotherapy drug treatment on mitochondrial trafficking, along with FA treated samples.

As expected PTX, VCR, and MMAE had a greater effect on degradation when focally treated on axons. Due to the neuronal cell structure, microtubule formation is key for nutrient transport [37]. Sensory neuron cells have an average axon length that can reach up to 1.5 m, requiring more microtubules to ensure proper nutrient support [25]. For this reason, the degeneration effect of PTX, VCR, and MMAE were expected to be higher when treated in regions with more microtubules. MMAE however shows similar results for cell body and axonal treatment. It is believed that MMAE site of action may be difficult to determine due to its high toxicity level [17]. MMAE has a similar mechanism to PTX and

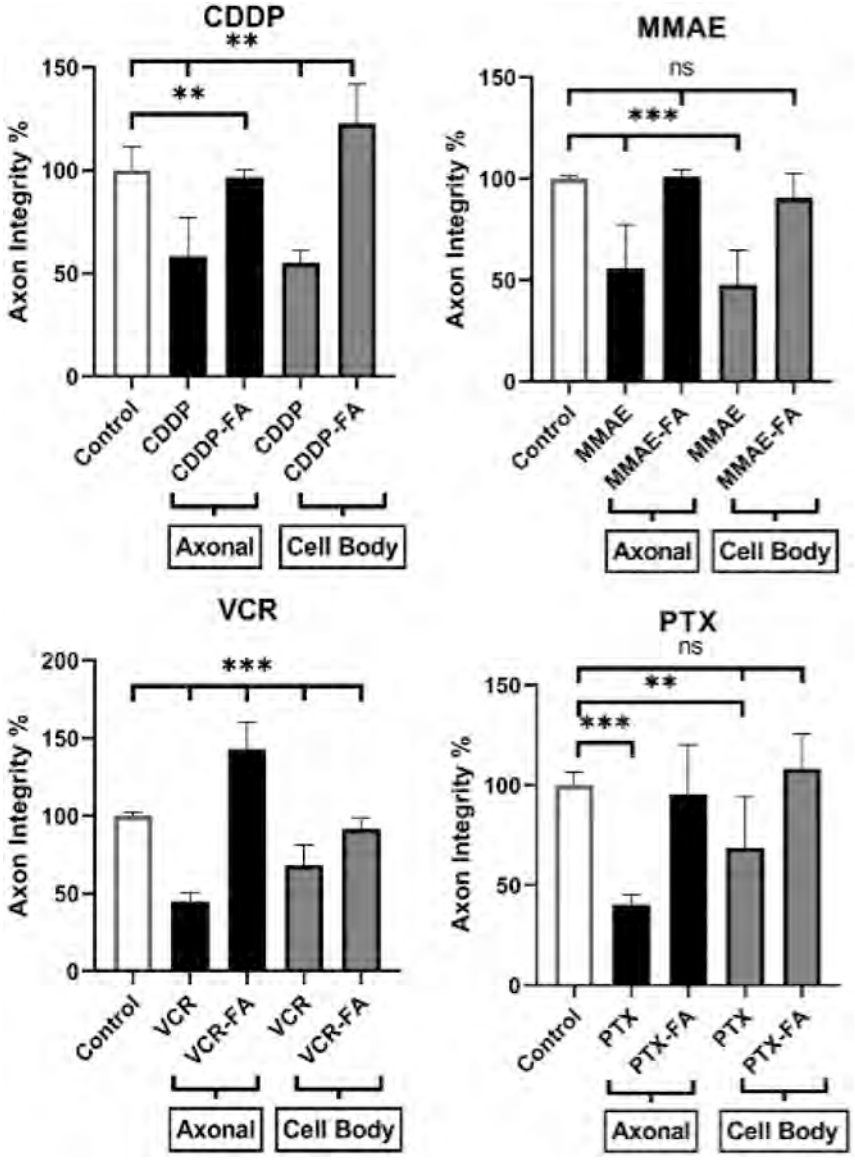

Fig. 5. Neuroprotective effect of FA on cell body and axonal administration. Each graph in this figure represents axonal and cell body administration of each drug, and corresponding FA treatment. All samples were normalized and calculated as a percentage of axonal integrity compared to their respective control samples. Triplicate samples were statistically processed. CDDP (top left), MMAE (top right), VCR (bottom left), PTX (bottom right). $^{***}p < 0.001$, $^{**}p < 0.01$.

VCR and shows great neuroprotection with the treatment of FA, however due to the high toxicity, it is difficult to specifically determine the site of action given the concentrations. Further research regarding the high toxicity response of MMAE is needed to conclude this finding in detail. While for CDDP, the degeneration effect was greater when treated on the cell body side due to the site of action that targets DNA replication, where majority of the DNA is located in the cell body region. Understanding the site of action of the drugs allowed for a better understanding of the delivery mechanism of CIPN. FA was treated alongside each of the drugs to examine its neuroprotective site of action effect. Although FA was neuroprotective in all treatment methods, the higher effect of neuroprotection on axonal treated samples can be seen by Fig. 5. These results suggest that the use of FA as a treatment option for CIPN is more effective when focally treated at the site of injury.

To answer these questions, we presume that each of the drugs used, has the ability to negatively affect an aspect of mitochondrial trafficking within the axon and cell body [45]. PTX, VCR and MMAE typically affect microtubule formation, and structure leading to the degradation of these microstructures which in turn negatively affects mitochondrial transport in the axon [1,22]. Within the axon of a neuronal cell, the microtubules have the vital function of sending organelles to and from the tip of the axon, or growth cone. Without proper nutrient transport, we assume that the cell slowly dies due to the decrease in mitochondrial trafficking and therefore energy availability [7]. The lack of nutrient transport could be a groundbreaking mechanism for understanding why dying back neuropathy occurs in CIPN patients [31,35]. Although CDDP has a greater effect on the cell body than axons, we believe that CDDP also has the ability to target mitochondrial DNA within the axon, affecting energy transport, which in turn causes peripheral neuropathy. Based on CDDP's ability to cause degeneration when focally treated to axons, it must have a mechanism that targets within the axonal section of the neuron. Since mitochondria are the only organelle aside from the nucleus to have DNA, we believe CDDP has the ability to disrupt the mitochondrial DNA replication cycle [44]. To prevent against CIPN, it is imperative to develop a deeper understanding of the functional mechanism behind it. From this experiment, treatment methodologies can be created to focally target the site of action of CIPN. Considering that FA is neuroprotective against the 4 different chemotherapeutic drugs tested,

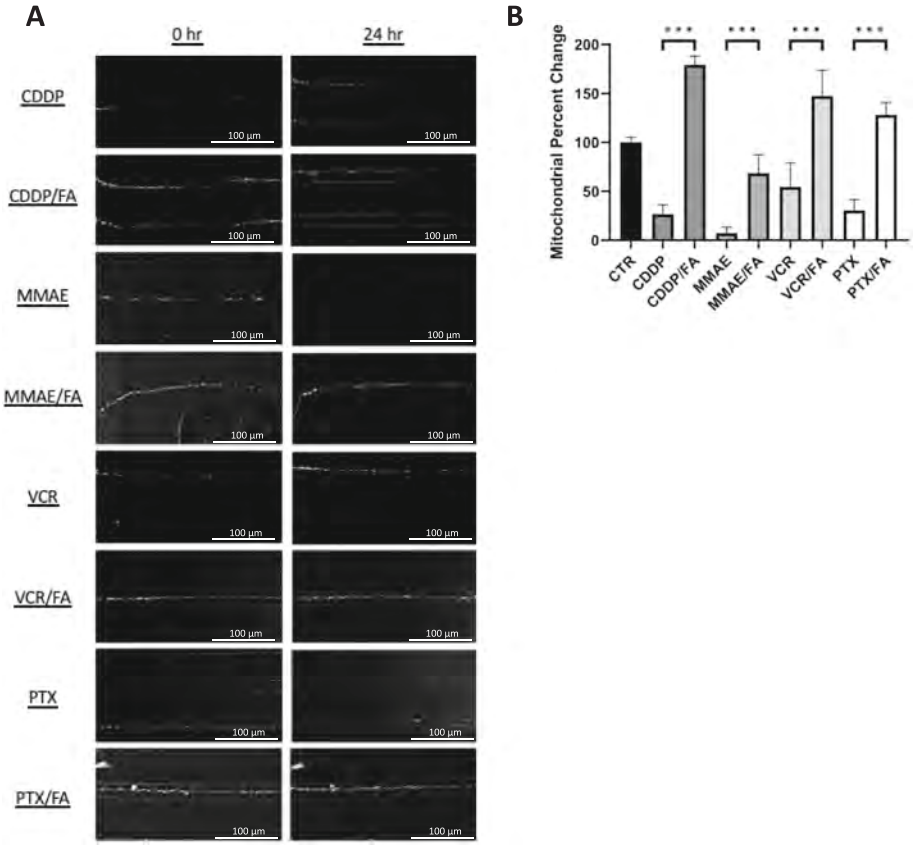

Fig. 6. Mitochondrial trafficking effect on axonal administration of drug treated DRG's. Panel A, Mito-Tracker Orange was used to fluorescently stain mitochondria for axonal treated samples. Mitochondria were imaged at 0 h and 24 h in correspondence with the site of action experiments. All drug concentrations used were the exact same for axonal treated samples. CDDP-10 μM, MMAE-1 μM, VCR-5 μM, PTX-5 μM. Likewise with FA treatment CDDP/FA-10 μM/20 nM, MMAE/FA-1 μM/20 nM, VCR/FA-5 μM/100 nM, PTX/FA-5 μM/10 nM. Mag. 63×. Panel B. Graphical representation of Mitochondrial Count analysis. Normalized percent change was calculated in triplicate samples. ***$p < 0.001$.

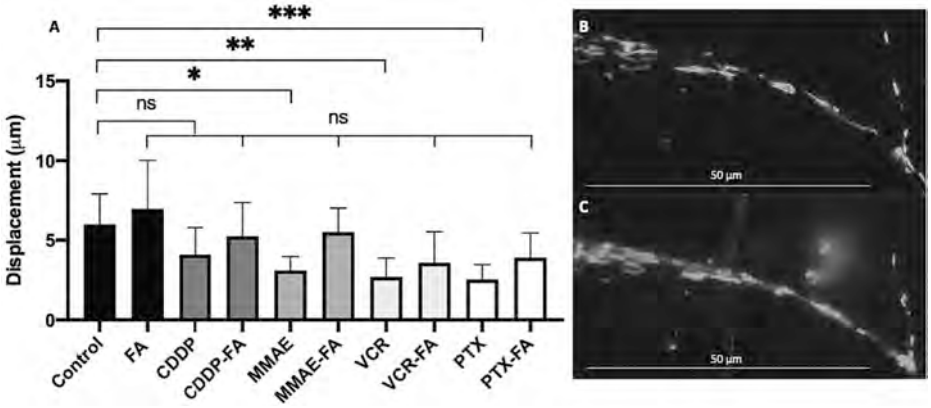

Fig. 7. Mitochondrial Displacement during a 30-min timelapse. Measured displacement using Fiji-ImageJ of mitochondria, where average displacement was calculated for each drug combination (A). Mitochondrial imaging was performed at 1-min intervals, where B, C are the first and last frame respectively for VCR/FA treatment. ***p < 0.001, **$p < 0.01$, *$p < 0.05$.

we believe that more research needs to be done to be able to identify whether axonal transport is a primary or secondary effect as a result of peripheral neuropathy.

5. Conclusions

Using the in vitro model, axonal susceptibility to chemotherapy drugs along with the neuroprotective mechanism of FA was analyzed using a compartmentalized microfluidic system (Figs. 2–5). Key results from this experiment show higher axonal susceptibility to microtubule targeting anti-cancer drugs and FA induced mitochondrial trafficking leading to neuroprotection [45]. The largest difference in axon integrity was shown with VCR, where cell body showed 31.80 % decrease in axon integrity while axonal treated sample showed 54.89 % decrease in axon integrity. This shows that for VCR, axons were 23.1 % more susceptible to axonal compared to cell body treatment. For the other drugs analyzed;

axons were 20.21 % more susceptible to *axonal treatment* for PTX, and 7.97 % more susceptible for MMAE. DRGs were more susceptible to *cell body treatment* 3.27 % for CDDP. For the samples analyzed, FA was found to have higher neuroprotection at the site of susceptibility. FA was 51.06 % more neuroprotective for axonal treated VCR, 15.24 % for PTX, and 10.51 % MMAE. On the other hand, FA was 26.66 % more neuroprotective for cell body treated CDDP. The results demonstrate important findings regarding the mechanism of CIPN and the neuroprotection by FA. Enhanced mitochondrial trafficking by FA proved increased neuroprotection for all CIPN models. Disregarding the mechanism of action for chemotherapy drugs, FA was found to be an effective neuroprotection strategy in vitro. Through subcellular mitochondrial trafficking analysis, we found that any form of enhanced trafficking may lead to increased neuroprotection against CIPN. The information gathered from this experiment can potentially lead to coadministration of chemotherapy drugs and further create a lower risk of CIPN. In this study, the site of action for each chemotherapy drug, CDDP, MMAE, VCR, and PTX was determined to help develop a better understanding of CIPN targeting mechanisms. FA was treated alongside each chemotherapy drug to identify the neuroprotective effect against CIPN, where FA was found to be neuroprotective for all drugs tested. This study found that treatment with FA led to an enhancement in the anterograde movement of mitochondria based on fluorescent imaging. Additional studies need to be performed to understand the fundamental mechanism and signaling pathways associated with enhanced mitochondrial trafficking. In vivo characterization of mitochondrial trafficking enhancement is necessary to fully consider FA as a neuroprotective treatment against CIPN.

CRediT authorship contribution statement

Bayne Albin: Conceptualization, Methodology, Validation, Formal analysis, Writing, Visualization. Khayzaran Qubbaj: Conceptualization, Methodology, Validation, Formal analysis, Writing. Arjun Prasad Tiwari: Methodology, Validation, Visualization, Supervision. Prashant Adhikari: Methodology, English correction. In Hong Yang: Conceptualization, Validation, Visualization, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

There are no conflicts of interests among authors.

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