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Amelioration of Cisplatin-Induced Experimental Peripheral Neuropathy by a Small Molecule Targeting p75^{NTR}

Amy Friesland^{1,2}, Zhiying Weng^{1,3}, Maria Duenas¹, Stephen M. Massa⁴, Frank M. Longo⁵, and Qun Lu^{1,2,*}

¹Department of Anatomy and Cell Biology, The Brody School of Medicine, East Carolina University, Greenville, North Carolina USA 27834

²Leo Jenkins Cancer Center, The Brody School of Medicine, East Carolina University, Greenville, North Carolina USA 27834

³School of Pharmaceutical Science and Yunnan Key Laboratory of Pharmacology for Natural Products, Kunming Medical University, Kunming China 650500

⁴Department of Neurology, University of California at San Francisco, San Francisco, California 94121

⁵Department of Neurology and Neurological Science, Stanford University, Stanford, California 94305

Abstract

Cisplatin is an effective and widely used first-line chemotherapeutic drug for treating cancers. However, many patients sustain cisplatin-induced peripheral neuropathy (CIPN), often leading to a reduction in drug dosages or complete cessation of treatment altogether. Therefore, it is important to understand cisplatin mechanisms in peripheral nerve tissue mediating its toxicity and identify signaling pathways for potential intervention. Rho GTPase activation is increased following trauma in several models of neuronal injury. Thus, we investigated whether components of the Rho signaling pathway represent important neuroprotective targets with the potential to ameliorate CIPN and thereby optimize current chemotherapy treatment regimens. We have developed a novel CIPN model in the mouse. Using this model and primary neuronal culture, we determined whether LM11A-31, a small-molecule, orally bioavailable ligand of the p75 neurotrophin receptor (p75^{NTR}), can modulate Rho GTPase signaling and reduce CIPN. Von Frey filament analysis of sural nerve function showed that LM11A-31 treatment prevented decreases in peripheral nerve sensation seen with cisplatin treatment. Morphometric analysis of harvested sural nerves revealed that cisplatin-induced abnormal nerve fiber morphology and the decreases in fiber area were alleviated with concurrent LM11A-31 treatment. Cisplatin treatment increased RhoA

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*Correspondence to Dr. Qun Lu, Professor of Anatomy and Cell Biology, The Brody School of Medicine, East Carolina University, Greenville, North Carolina USA 27834, 252-744-2844, luq@ecu.edu.

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activity accompanied by the reduced tyrosine phosphorylation of SHP-2, which was reversed by LM11A-31. LM11A-31 also countered the effects of calpeptin, which activated RhoA by inhibiting SHP-2 tyrosine phosphatase. Therefore, suppression of RhoA signaling by LM11A-31 that blocks proNGF binding to p75^{NTR} or activates SHP-2 tyrosine phosphatase downstream of NGF receptor enhances neuroprotection in experimental CIPN in mouse model.

Keywords

Chemotherapeutics; Peripheral neuropathy; Rho GTPases; p75^{NTR}; Kinases; phosphatases

1. Introduction

Cisplatin (cis-diamminedichloroplatinum), a platinum-based compound, is a widely used and valuable tool to suppress cancer cell proliferation and promote remission from various solid tumors. The canonical cisplatin mechanism of action involves induction of inter- and intra-strand DNA cross-links which ultimately results in the apoptosis of rapidly dividing cells (Huang et al., 1995). Unfortunately, cisplatin's cytotoxic effects are not limited to neoplastic tissue, as it frequently disrupts normal renal, gastrointestinal, hematologic, and peripheral nervous system function.

Peripheral neurotoxicity is the most common dose-limiting side effect of cisplatin, and often leads to dose reduction or complete cessation of effective treatment protocols. It is estimated that up to 50% of patients receiving cisplatin develop some degree of peripheral neurotoxicity (Gutiérrez-Gutiérrez et al., 2010; Strumberg et al., 2002; van der Hoop et al., 1990) as a consequence of treatment. Neuropathies associated with cisplatin are predominately sensory in nature and tend to present in a “glove and stocking” fashion as numbness, tingling, and pain that initiates in the distal extremities (Jaggi and Singh, 2012; Kaley and Deangelis, 2009). Incidence and severity of cisplatin-induced peripheral neuropathy (CIPN) is dose-dependent (Gregg et al., 1992) and may persist or resolve following treatment completion (van der Hoop et al., 1990). The highest levels of cisplatin tissue accumulation occur within the dorsal root ganglion (DRG) where it preferentially binds DNA (McDonald et al., 2005; Park et al., 2008). Although it is apparent that there must be additional targets aside from mitotic DNA replication, as neurons are not a proliferating population of cells, cisplatin's precise neurotoxic mechanism remains uncertain. Thus, it has become important not only to understand alternative cisplatin targets but to also explore signaling pathways for potential intervention.

In recent years, it has been well established that RhoA, a small GTPase, has increased activity following trauma in several models of neuronal injury (Brabeck et al., 2004; Cheng et al., 2008; Dergham et al., 2002; Dubreuil et al., 2003; Dubreuil et al., 2006). Additionally, inhibition of the RhoA pathway has been shown to be effective in experimental models of Alzheimer's disease, stroke, neuropathic pain and inflammatory reactions (Tatsumi et al., 2005; Walters et al., 2002; Zhou et al., 2003). *In vitro* studies from our laboratory have demonstrated that Rho pathway inhibition, either upstream with a p75 neurotrophin receptor (p75^{NTR}) small molecule ligand (LM11A-31, Massa et al., 2006) or downstream with a selective p160^{ROCK}/Rho kinase inhibitor (Y-27632), facilitates neuronal recovery in

neuronal cultures treated with cisplatin (James et al., 2008). Subsequent *in vivo* studies, using a CIPN mouse model, demonstrated that Y-27632 inhibited cisplatin-induced RhoA pathway activation and promoted recovery from peripheral neuropathy (James et al., 2010). These findings highlight the potential importance of RhoA signaling in CIPN and raise the possibility that a clinically viable way to inhibit RhoA activation might serve to inhibit neuronal toxicity and thereby enhance current cisplatin treatment paradigms.

LM11A-31 has recently been approved by the FDA for Phase I pharmacokinetic and safety studies in normal subjects and these studies are underway. Therefore, we tested the hypothesis that administration of this compound to the CIPN mouse model might prevent cisplatin-induced RhoA activation and peripheral neuropathy. Using this model, we show that cisplatin-induced decreases in peripheral sensory nerve function can be prevented with LM11A-31. This study reveals the importance of RhoA in the development of CIPN and suggests a potential site of intervention that could be exploited clinically for patients undergoing chemotherapy.

2. Materials & Methods

2.1. Pharmacological treatments

Forty-Four (22 male; 22 female) age-matched C57/BL6 mice (3 months; Charles River, Wilmington MA) weighing an average of 24 g were obtained. Mice were treated with either 6 µg/g body weight cisplatin (Sigma Co, St. Louis MO) or 200µL 0.9% saline (Hospira, Lake Forest IL) by intraperitoneal injection every 14 days for a 10 week treatment period (n=10; 5 male & 5 female mice/ group). In addition to cisplatin (6 µg/g body weight), two additional groups of animals received either a low dose (25 µg/g body weight) or a high dose (50 µg/g body weight) of LM11A-31 by intraperitoneal injection once every 24 hours for the 10 week treatment period (n=12; 6 male & 6 female mice/group). Mice were observed daily throughout the treatment for hair and weight loss. All animal experiments and testing were approved by the East Carolina University Animal Care and Use Committee.

2.2. Von Frey/ Semmes-Weinstein sensory threshold testing of hind paws

All animals were subjected to Von Frey/Semmes-Weinstein monofilament sensory threshold testing of the hind paws as previously described (James et al., 2010) every two weeks for the 10 week treatment period. Briefly, animals were placed within a clear plastic barrier upon an elevated screen and allowed to acclimate for 10 to 15 minutes. Von Frey/Semmes-Weinstein monofilaments (Stoeling Co., Wood Dale, IL) were applied to the point of bending onto the plantar surface of left and right hind paws. Monofilament testing proceeded from the smallest diameter until the applied monofilament elicited a response. Responses were recorded as positive when the animal responded in 3 out of 5 applications. Differences between treatment groups were determined by *t*-test analysis using SigmaPlot 10.0 (Systat Software Inc., San Jose CA).

2.3. Tissue preparation

Animals were deeply anesthetized with 3% isoflurane vapor and euthanized by cervical dislocation. Tissues (dorsal root ganglion, sural and sciatic nerves) were rapidly removed

and either fresh frozen on liquid nitrogen (for protein analysis) or fixed in Zamboni's fixative (2% paraformaldehyde, 0.5% picric acid, and 0.1% phosphate buffer) overnight. Tissue samples were then washed with phosphate-buffered saline and cryoprotected overnight in 30% sucrose in PBS at 4°C. Tissues were then embedded in OCT and rapidly frozen with chilled isopentane. 5 µm thick cryostat sections were affixed to Superfrost slides (Fisher Scientific, Pittsburgh PA).

2.4. Immunofluorescent analysis of peripheral nerve tissue

For immunostaining, sections were incubated with 0.2% Triton X-100 for 10 minutes at room temperature, 20 minutes at room temperature with 100mM glycine and blocked 30 minutes at 37°C with 10% bovine serum albumin (BSA). Primary antibodies (rabbit antibody against total RhoA at 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit antibody against phosphorylated RhoA at 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) were all incubated overnight at 4°C. Immunofluorescent images were captured and analyzed with a Zeiss Axiovert S100 fluorescent light microscope (Carl Zeiss, Thornwood, NY). Pixel intensity was measured at 50 random points of each sample and averaged to generate average pixel intensity for each treatment group using MetaMorph™ 4.6 imaging software systems (Molecular Devices, Sunnyvale, CA). n= 3 sural nerve cross sections harvested from 3 different mice/ treatment group; Saline-treated mice were used as controls.

2.5. Morphological analysis of peripheral nerve tissue

Morphometric evaluation of phase contrast images of sural nerve cross sections, captured at 63X with a Zeiss Axiovert S100 fluorescent light microscope (Carl Zeiss, Thornwood, NY), was conducted using MetaMorph software (Sunnyvale, CA). Due to natural size variation in sural nerves, morphometric analysis was carried out on three randomly selected 40 X 40 µm regions selected from each of three cross sections for each nerve and averaged to obtain data for each sural nerve evaluated. Myelinated fiber (axon + myelin), axon, and myelin areas were measured with image analysis software as described in James et al (10). Based on the total area of the region counted (1600 µm²) percentages of area occupied by axons, myelin, and total fibers were calculated. Additionally, after subtracting the total fiber area from the total area of the region, the percentage of endoneurial space that was unoccupied by myelinated nerve fibers was determined. Two-side *t*-test analysis was performed using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA) to determine differences between drug treatment groups. n= 6 sural nerves/ treatment group. Any null hypothesis with the probability level less than 95 % was rejected.

2.6. Protein analysis of peripheral nerve tissue

Due to the inability to obtain sufficient protein from sural nerves, flash frozen sciatic nerves were utilized for protein analysis. Harvested sciatic nerves were frozen on liquid nitrogen, crushed, and homogenized in a buffer consisting of 50mM Tris pH 7.4, 50mM sodium chloride, 5 mM magnesium chloride, 1 mM dithiothreitol (DTT), and 1% Triton X-100, and supplemented with protease inhibitors (1mM phenylmethanesulfonylfluoride (PMSF), 10mM sodium pyrophosphate, 20mM sodium fluoride, 1mM sodium orthovanadate, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)). After determination

of protein concentrations, homogenates were equally loaded into and 18% tris-glycine gel (Invitrogen, Carlsbad CA), transferred to PVDF membrane (Milipore, Billerica MA) and subjected to Western blot analysis. Blots were blocked and probed with the following antibodies: mouse antibody against total RhoA at 1:400 (Cytoskeleton, Denver CO); rabbit antibody against phosphorylated RhoA at 1:500 (Santa Cruz Biotechnology, Santa Cruz CA); rabbit antibody against total SHP-2 at 1:500 (Milipore, Billerica, MA); and phosphorylated SHP2 (Y542) at 1:500 (Cell Signaling Technology, Danvers MA). GAPDH antibody (mouse, 1:2000; Calbiochem, San Diego CA) was used as a loading control.

2.7. Primary neuronal cultures

Primary cortical neurons were isolated from 18-day timed pregnant mice as previously described (James et al., 2008). Neurons were cultured for 5 days *in vitro* (DIV) in B-27 supplemented Neurobasal media (Invitrogen Carlsbad, CA) and then treated for 24 hours with either cisplatin (8 µg/mL) or PBS. LM11A-31 or PBS was then applied at 6 DIV at a final concentration of 100 nM for an additional 24 hours. Finally, at 7 DIV, selected cultures were treated with calpeptin for 30 minutes (1 unit/mL; Cytoskeleton, Denver CO). Neurons were then fixed for 15 minutes in 4% paraformaldehyde.

2.8. Neuronal morphological analysis

Neuronal actin-based structures were identified with fluorescein-phalloidin (Molecular Probes, Eugene OR) and nuclei were labeled with Hoechst 33258 (Sigma). Neuronal images were captured using a Zeiss Axiovert S100 (Carl Zeiss, Thornwood NY) microscope. Morphometric analyses were then performed using the MetaMorph™ 4.6 imaging software system (Universal Imaging Corp, West Chester, PA) as previously described (James et al., 2008; Jones et al., 2004). Briefly, branch numbers were determined by applying the MEASURE COUNT OBJECT function. Branches were scored when they were longer than 10 µm. Processes shorter than 10 µm were designated as sprouts, and not counted as branches. The imaging system was calibrated so that dendrite length measurements were true values. Two-sided *t*-test analysis was performed using SigmaPlot 10.0 (Systat Software Inc., San Jose CA) to determine differences between drug treatment groups. *n* = 3 representative neurons/treatment group. Any null hypothesis with the probability level less than 95 % was rejected.

2.9. Neuronal RhoA immunofluorescence

Primary cortical neurons on poly-L-lysine coated coverslips, treated as previously described and fixed at 7 DIV, were incubated with 0.2% Triton X-100 for 10 minutes at room temperature, 20 minutes at room temperature with 100mM glycine and blocked for 30 minutes at 37°C with 10% bovine serum albumin (BSA). Primary antibodies (rabbit antibody against total RhoA at 1:100 and rabbit antibody against phosphorylated RhoA at 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) were incubated for one hour at room temperature. Immunofluorescent images were captured and analyzed with a Zeiss Axiovert S100 fluorescent light microscope (Carl Zeiss, Thornwood, NY). Pixel intensity was measured at 50 random points of each sample and averaged to generate average pixel

intensity for each treatment group using MetaMorph™ 4.6 imaging software systems (Molecular Devices, Sunnyvale, CA). n= 3 representative neurons/ treatment groups.

2.10. Analysis of protein expression in neuronal lysates

Mouse primary cortical neurons were treated as previously described, lysed at 7 DIV with RIPA buffer supplemented with protease inhibitors (1mM phenylmethanesulfonylfluoride (PMSF), 10mM sodium pyrophosphate, 20mM sodium fluoride, 1mM sodium orthovanadate, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)), and subjected to Western blot analysis. Blots were blocked and probed with anti-phosphorylated RhoA 1:500 (Santa Cruz Biotechnology, Santa Cruz CA) and anti-rabbit phosphorylated SHP-2 (Y542) 1:500 (Cell Signaling Technology, Danvers MA). Blots were also probed with anti-total RhoA and SHP-2 as loading controls.

3. Results

3.1. Cisplatin-induced decline in sensory function can be prevented with concurrent use of LM11A-31

CIPN was induced in age-matched C57/BL6 mice by intraperitoneal administration of cisplatin once every two weeks for a ten week treatment period (5 successive doses). Such doses are clinically relevant and represent a high, yet tolerable, dose successfully used to induce neuropathy in mice (James et al., 2010; Verdú et al., 1999). In addition to cisplatin, two additional, selective groups of mice received, at the same time, either a high or low IP dose of the p75^{NTR} receptor ligand, LM11A-31. This compound targets the p75^{NTR} receptor (Massa et al., 2006; Longo and Massa, 2008), which is known to lie upstream of RhoA activation and modulate Rho GTPase activity (James et al., 2008). Saline-treated mice were maintained as experimental controls. Mice were observed daily for clinical signs of chemotherapy treatment such as hair and weight loss. All mice receiving cisplatin showed consistent weight loss over the course of treatment, as well as increased hair loss. Any mice displaying any signs of discomfort, pain, or weight loss exceeding 20% of original body weight were euthanized.

In order to assess peripheral nerve function in both control and experimental groups, Von Frey/Semmes-Weinstein monofilament testing was performed as previously described (James et al., 2010; Ta et al., 2009). Briefly, Von Frey/Semmes-Weinstein monofilaments of varying diameters, which correspond to force, were sequentially applied to the plantar surface of the hindpaw until positive responses were elicited. Positive responses included both hind paw withdrawal and licking. Clinically, similar testing is frequently employed to screen patients with suspected diabetic or chemotherapy-induced peripheral neuropathies (Grisold et al., 2012; Feng et al., 2009).

In the current experiment, touch perception analysis with Von Frey/Semmes-Weinstein monofilaments showed a significant increase ($p= 0.04$, relative to saline) in force required to elicit a response, indicative of a decline in sensory nerve function, in the cisplatin treated group after 6 weeks (Fig. 1). However, addition of LM11A-31 at either high or low doses prevented these deficits in touch perception, as seen by significant reductions ($p= 0.02$, relative to cisplatin) in force applied to the hindpaw. In non-cisplatin treated mice, neither

LM11A-31 doses showed responses that were significantly different from saline over the 10 week treatment period.

3.2. LM11A-31 treatment reduces abnormal sural nerve fiber morphology induced by cisplatin

The rodent hindpaw, as well as the human foot, is innervated by branches of the sciatic nerve (tibial, common peroneal (fibular) and sural). The predominately sensory sural nerve provides innervation to the lower hind leg, heel, and plantar surface of the hindpaw. Therefore, sural nerves were harvested post treatment, fixed, frozen and 5 μ m cross-sections were subjected to further analysis. Through phase contrast microscopy, which highlights the myelin sheath as dark ring-like structures surrounding the inner axon, differences in nerve fiber morphologies can be observed between control and cisplatin-treated groups. As shown in Figure 2A, saline-treated nerve fibers displayed round morphologies that were well myelinated (red arrow). However, in cisplatin-treated nerves abnormally shaped nerve fibers (yellow arrows) and decreases in the degree of myelination (red arrows) were frequently observed, which is consistent with our previous data (James et al., 2010). Administration of high doses of LM11A-31 to cisplatin-treated mice alleviated myelin sheath reduction (red arrow) and fibers maintained a rounded fiber morphology closely resembling that of saline. Interestingly, nerve fibers that received a lower dose of LM11A-31 maintained a high degree of myelination but, like cisplatin fibers, anomalous flattened and infolded morphologies were consistently observed (yellow arrows), suggesting a dose-related effect.

Due to natural variation in nerve sizes between animals, randomly selected 40 X 40 μ m regions of sural nerve cross sections were used to collect and quantify morphological data (Fig.2B-F). All sural nerve sections were derived proximally to the sciatic bifurcation and displayed a single fascicle. Comparisons between the percentage of the total area occupied by axons, myelin and total fibers (myelin plus axon; Fig. 2B, C and D, respectively) showed a reduction in cisplatin-exposed nerves which was prevented by treatment with either dose of LM11A-31 ($p=0.05$). Additionally, a 10% increase in percentage of the total area not occupied by fibers observed in cisplatin nerves ($p=0.05$) was diminished with either LM11A-31 doses ($p=0.05$; Fig.2E). Importantly, quantification of nerve fiber density treated concurrently with LM11A-31 and cisplatin was not significantly different from that found in saline treated mice. G-ratios, which describe the degree of myelination of a nerve fiber as a ratio between axonal diameter/fiber diameter (Rushton, 1951), were also calculated. Measurements revealed G-ratios within the optimal range (0.47–0.6) for the sural nerve in all four treatment groups (Chomiak and Hu, 2009; Fig.2F). Cisplatin treatment led to a small decrease in G-ratio compared to saline but this difference did not reach significance. With both high and low LM11A-31 doses, the G-ratio was significantly increased compared to that found with cisplatin exposure alone, while ratios in LM11A-31- and saline-treated nerves were not significantly different. This data suggests that p75 NTR receptor may play a role in myelin regulation (Cosgaya et al., 2002; Song et al., 2006).

3.3. LM11A-31 inhibits cisplatin-induced increase in phosphorylated RhoA but promotes SHP-2 phosphorylation in peripheral nerves

To determine and compare RhoA activity levels between treatment groups we performed immunofluorescent analysis of frozen sural nerve cross sections with antibodies detecting total levels of RhoA (tRhoA) and phosphorylated RhoA (pRhoA). Phosphorylation at serine 188 is an important site for negative regulation of RhoA by cAMP and cGMP-dependent kinases (Dong et al., 1998; Ellerbroek et al., 2003; Sawada et al., 2001). Therefore, decreases in pRhoA are indicative of an increase in the levels of active (GTP-bound) RhoA. Immunofluorescent data revealed only a slight increase in tRhoA activity in cisplatin nerves compared to controls, while both doses of LM11A-31 showed an overall reduction in tRhoA activity (Fig.3A, top panel). Quantification of pixel intensity in Fig.3B confirms the visual comparison and demonstrates LM11A-31 not only significantly reduces tRhoA activity compared to cisplatin ($p=0.05$), but also to saline ($p=0.0001$). pRhoA immunostaining (Fig. 3A, bottom panel), on the other hand, reveals a dramatic 32% pRhoA decrease in cisplatin versus saline nerves ($p=0.0001$, Fig. 3C), suggesting an increase in active RhoA levels. Addition of high doses of LM11A-31 was associated with a 40% pRhoA activity increase relative to cisplatin ($p=0.0001$), a level nearing that of saline controls. However, the lower LM11A-31 dose was only minimally effective in preventing cisplatin-induced pRhoA decreases with only a 2% increase in pixel intensity versus cisplatin only nerves (Fig.3C). Interestingly, this data implies that tRhoA activity can be inhibited similarly regardless of our high or low dose, but LM11A-31 is less sensitive at lower doses for pRhoA activity inhibition. When expressed as a ratio of pRhoA to tRhoA the pixel intensity quantification more clearly describes the activity level of RhoA due to treatment (Fig. 3D). These data demonstrate a significant 40% increase in RhoA activity due to cisplatin which could be significantly inhibited by the addition of high (65%; $p=0.0002$) and low (37% $p=0.04$) LM11A-31 doses. Such results not only support our hypothesis that RhoA activity is increased due to cisplatin treatment, but suggest that treatment with LM11A-31, in a dose-dependent manner, inhibits RhoA activation.

In order to further confirm RhoA's role in CIPN we assessed protein expression in fresh frozen sciatic nerve samples. Due to insufficient protein concentrations obtained from mouse sural nerves, we utilized the larger sciatic nerve from which the sural nerve branches. The tRhoA expression appeared to be unchanged in the sciatic nerve, regardless of treatment (Fig. 4, first panel). However, reflecting pRhoA immunofluorescent results, cisplatin decreased pRhoA levels and concurrent use of LM11A-31 lead to levels similar to that of controls (Fig.4, second panel). SHP-2 tyrosine phosphatase, downstream of NGF receptor tyrosine kinases, is known to modulate RhoA signaling. Additional analysis of both phosphorylated (Fig. 4, third panel) and total SHP-2 phosphatase (Fig. 4, fourth panel) levels were performed. Results demonstrated cisplatin-associated increased levels of phosphorylated SHP-2, which was correlated with increased RhoA activity. When used in conjunction with cisplatin, LM11A-31 treatment was able to abrogate SHP-2 phosphorylation. However, similarly to tRhoA levels (Fig. 4, first panel), total levels of SHP-2 (Fig.4, fourth panel) were not altered by either cisplatin or LM11A-31.

3.4. LM11A-31 and direct SHP-2 phosphatase inhibition can modulate cisplatin-induced neurodegeneration

RhoA has been well established as a negative regulator of neuronal branching (Ahnert-Hilger et al., 2004; Bito et al., 2000; Threadgill et al., 1997). In order to further analyze the effects of cisplatin and LM11A-31 on RhoA and RhoA-mediated processes *in vitro*, a primary cortical neuronal culture system was established in which present neurite branching development can be characteristically observed. Typical neuronal development begins with axonal extension at 3 days *in vitro* (DIV) and establishment of dendritic processes from 5-7 DIV (Dotti et al., 1988; James et al., 2008). Therefore, cortical neurons were cultured for 5 DIV, treated with cisplatin, and after 24 hours, LM11A-31 was then applied for an additional 24 hours. Additionally, at 7 DIV, RhoA was constitutively activated in selected cells with a SHP-2 phosphatase inhibitor, calpeptin, for 30 minutes (1 unit/mL). Tyrosine dephosphorylation of p190-B RhoGAP modulated by SHP-2 phosphatase has been shown to function as a constitutive RhoA activator (Kontaridis et al., 2004; Sordella et al., 2003). Under our experimental condition, TUNEL staining did not show significant differences among all treatment groups (data not shown). Additionally, Hoechst 33258 staining did not show significant nuclear condensation or morphological alterations among all treatment groups indicating that all cells were viable.

As depicted in Fig 5A (upper panel), immunofluorescent analysis of actin-based structures reveals a distinct reduction in branching due to treatment with either calpeptin or cisplatin. Quantification of branch number (Fig. 5B), sprout number (Fig. 5C), and branch length (Fig. 5D) demonstrated significant reductions due to cisplatin treatment. However, RhoA activation through SHP-2 phosphatase resulted in less dramatic (not significant) reductions in both branch (Fig. 5B) and sprout (Fig. 5C) numbers. In contrast, addition of LM11A-31 to cisplatin-treated cultures increased branch numbers (Fig 5B), sprout numbers ($p<0.03$; Fig. 5C), and branch length ($p<0.02$; Fig. 5D). Concurrent treatment of LM11A-31 and calpeptin resulted in a significant increase in branch number ($p<0.05$; Fig. 5B), while sprouting and branch length remained unchanged.

In order to measure RhoA phosphorylation in treated neurons, pRhoA immunostaining was performed. Total RhoA levels (Fig. 6A; upper panel) were shown to be similar in cisplatin and control neurons, while calpeptin treatment significantly increased the total level of RhoA (Fig. 6B; $p<0.005$). Addition of LM11A-31 to both cisplatin and calpeptin-treated cells resulted in significant decreases in tRhoA expression compared to each treatment alone (Fig. 6B). As shown in Fig. 6A (lower panel) and quantified in Fig. 6C, results revealed a significant increase in pRhoA expression due to both calpeptin ($p<0.01$) and cisplatin ($p<0.0006$) application relative to non-treated controls. The addition of LM11A-31 significantly inhibited the increase elicited by cisplatin ($p<0.003$). The same inhibitory trend from LM11A-31 can be observed for these increases elicited by calpeptin, although not significant. The ratio of pRhoA to tRhoA pixel intensity revealed expected increases in the expression of RhoA phosphorylation in both calpeptin ($p<0.0006$) and cisplatin-treated ($p<0.002$) neurons that was attenuated with addition of LM11A-31 to cisplatin-treated ($p<0.009$) neurons, but not those treated with calpeptin (Fig. 6D). These results not only further support RhoA as a negative regulator of neurite branching, but confirm our *in vivo*

results suggesting that LM11A-31-mediated RhoA inhibition can preserve neuronal structure during cisplatin treatment.

Additional analysis of protein expression in lysates prepared from primary neuronal cultures treated with cisplatin, calpeptin and LM11A-31 were performed. As depicted in Fig. 6E decreases in pRhoA expression, indicative of increased levels of active RhoA, were observed in cisplatin-treated lysates which could be countered by LM11A-31 application. However, calpeptin treatment did not result in major reductions in pRhoA expression. Additionally, co-treatment of neuronal cultures with LM11A-31 and calpeptin did not display clear inhibitory actions on protein expression levels. Phosphorylated SHP-2 (pSHP-2) expression, which is indicative of inhibited SHP-2 phosphatase activity and increased RhoA activation, was shown to be significantly decreased due to both cisplatin ($p < 0.02$) and calpeptin-treatment ($p < 0.03$; Fig. 6F). However, LM11A-31 was able to attenuate cisplatin-induced pSHP-2 reductions but not calpeptin-induced pSHP-2 reductions. These results indicate that at the neuronal culture level, there are likely two independent mechanisms of RhoA activation, with one through p75^{NTR} and the other through calpeptin-induced inhibition of SHP-2 phosphatase.

4. Discussion

Peripheral neuropathy remains a debilitating, dose-limiting side effect of many platinum-based chemotherapeutics. To date, multiple avenues of neuroprotective intervention have been investigated with concurrent cisplatin use including vitamin E (Pace et al., 2003; Pace et al., 2007; Pace et al., 2010), Glutathione (Cascinu et al., 1995; Smyth et al., 1997) and Org 2766, an adrenocorticotrophic hormone analogue (Koeppen et al., 2004; van der Hoop et al., 1990a). However, clinical success has been minimal; leaving dose reductions or alterations in dose scheduling the only option for patients.

In the current study, we show elevation of the small GTPase RhoA is correlated with increased incidence of peripheral neuropathy in a mouse model of CIPN. Additionally, upstream inhibition of RhoA with LM11A-31, a p75^{NTR} receptor ligand, could not only suppress RhoA upregulation in cisplatin-treated mice, but prevent cisplatin-induced decline in peripheral nerve function seen using Von Frey/Semmes-Weinstein monofilament testing. Importantly, abnormal sural nerve fiber morphologies associated with cisplatin treatment was markedly reduced with the administration of LM11A-31 at both high and low doses. Within *in vivo* studies both doses were similarly effective, indicating that lower doses can be addressed in future studies. Additionally, our *in vivo* findings were consistent with our *in vitro* studies, suggesting a novel neuroprotective target that could be exploited during cisplatin chemotherapy.

p75^{NTR} receptor acts as a displacement factor that releases RhoA from Rho GDP-dissociation inhibitor (Yamashita and Tohyama, 2003). This activation of RhoA has a key role in inducing inhibition of axon growth (Ahnert-Hilger et al., 2004; Kranenburg et al., 1997). Our studies support a model that besides its inhibitory action on DNA synthesis, cisplatin elicits cellular stress leading to p75^{NTR} receptor dissociation from RhoA, thereby promoting RhoA activation and signaling to Y-27632-sensitive p160^{ROCK}/Rho kinase.

Increased RhoA/ROCK signaling results in axon retraction and repulsion (Nakayama et al., 2000; Fig 7). Furthermore, cisplatin-induced cellular stress suppresses SHP-2 tyrosine phosphatase, which also activates RhoA. Calpeptin-sensitive inhibition of SHP-2 activates RhoA and attenuates neurite branching and extension, further supporting this model. Notably, cisplatin-induced increases in RhoA activity and decreases in tyrosine phosphorylation of SHP-2 can be reversed by LM11A-31 treatment. LM11A-31 also countered the trends of calpeptin in activating RhoA and reducing neuronal sprout numbers. Therefore, LM11A-31 is a small molecule that can effectively reduce the impact of experimental CIPN.

Identification of RhoA signaling as a potential neuroprotective target in CIPN has important implications in terms of both nerve protection and efficacy of cancer therapy. Studies have shown that inhibition of RhoA downstream effector p160^{ROCK}/Rho kinase by Y-27632 attenuated tumor growth, indicating that suppression of RhoA signaling may be used as an adjuvant to promote cisplatin chemotherapeutic effects (Routhier et al., 2010). As demonstrated here, employment of a RhoA inhibitor in the treatment regimen may also allow cisplatin to be effective at the lower dosages, further reducing the risks of peripheral neuropathy. The identification of the RhoA upstream modulator p75^{NTR} and its modulator LM11A-31 provides yet additional important evidence that RhoA suppression is a potential site of intervention to circumvent CIPN. Future studies will assess the neuroprotective effects of LM11A-31 in tumor-bearing mouse models as a significant step towards its clinical application. A form of LM11A-31 is currently in a Phase 1 pharmacokinetic and safety study in normal subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We have developed a novel CIPN model in the mouse where LM11A-31, a small-molecule, orally bioavailable ligand of the p75 neurotrophin receptor (p75^{NTR}), can modulate Rho GTPase signaling and reduce CIPN.
- Von Frey filament analysis of sural nerve function and morphometric analysis were used to determine neuroprotective function of LM11A-31 treatment.
- We found that cisplatin treatment increased RhoA activity accompanied by the reduced tyrosine phosphorylation of SHP-2, which was reversed by LM11A-31.
- LM11A-31 also countered the effects of calpeptin, which activated RhoA by inhibiting SHP-2 tyrosine phosphatase.
- Our studies demonstrated that suppression of RhoA signaling by LM11A-31 that blocks proNGF binding to p75^{NTR} or activates SHP-2 tyrosine phosphatase downstream of NGF receptor enhances neuroprotection in experimental CIPN in mouse model.

Figure 1. Cisplatin-induced decline in sensory function can be prevented with concurrent use of LM11A-31

Peripheral nerve function was analyzed by mechanical (touch) stimulus perception using Von Frey/ Semmes-Weinstein monofilaments. Results showed that cisplatin induced a decrease in sensory function over the course of treatment that could be prevented by either high or low doses of LM11A-31. Values represent mean $\pm$ S.D. $p < 0.04$ *, relative to saline; $p < 0.02$ Δ , relative to cisplatin. $n = 10$ animals/ treatment group saline and cisplatin; $n = 12$ animals/ treatment group LM11A-31 high and low dose.

Figure 2. LM11A-31 treatment reduces abnormal sural nerve fiber morphology induced by cisplatin

Sural nerve cross sections were analyzed by phase-contrast microscopy (63X Upper and Lower Panels). (A Upper Panel) Cisplatin altered sural nerve morphology (yellow arrows) and myelination (red arrows) in comparison to saline treatment. LM11A-31 treatment reversed the cisplatin-induced abnormally shaped nerve and reduced myelination. A lower panels demonstrate enlargements of myelin sheath indicated in the upper panel by red arrows. Quantification of sural nerve axon (B), myelin (C), fibers (axon + myelin, D), unoccupied (E) percent of areas in randomly selected 40 μm^2 regions, and G-ratio (ratio of

axonal diameter to the total fiber diameter which then reflects the level of myelination of a nerve fiber, F) of sural nerve cross sections. Results indicate a reduction in the percent of area occupied by axons (B), myelin (C) and fibers (D) in the cisplatin treated group and a significant recovery with LM11A-31 treatment. Additionally, the percent of space unoccupied by nerve fibers (E) was significantly increased in cisplatin treated sural nerves, which could be prevented by addition of LM11A-31. G-ratio (F) calculations revealed the degree of myelination is significantly increased with addition of LM11A-31 relative to cisplatin. Values represent mean $\pm$ S.D. $p < 0.05$ **, relative to saline; $p < 0.05$ *, relative to cisplatin. $n=6$ regions/ treatment group.

Figure 3. LM11A-31 inhibits cisplatin-induced increase in phosphorylated RhoA in the sural nerve

Total RhoA (tRhoA) and phosphorylated RhoA (pRhoA) immunofluorescent analysis of 5 μ m sural nerve cross sections. (A, lower panel) Results revealed an increase in the levels of active RhoA due to cisplatin treatment as indicated by a decrease in pRhoA immunostaining. However, cisplatin treatment did not alter the expression of total RhoA levels (A, upper panel). Concurrent treatment with LM11A-31 showed an increase in pRhoA and a decrease in tRhoA immunostaining which is indicative of RhoA inhibition. Quantification of pixel intensity of tRhoA (B), pRhoA (C) and the ratio of pRhoA pixel intensity to tRhoA pixel intensity (D) were determined. Values represent mean $\pm$ S.D.; *, relative to saline; Δ , relative to cisplatin. n=4 nerves/ treatment group.

Figure 4. Increased pRhoA and pSHP2 protein expression due to cisplatin treatment can be inhibited by a high dose of LM11A-31

Mouse sciatic nerves were harvested, homogenized and subjected to Western blot analysis. Blots were then probed with total RhoA (tRhoA), phosphorylated RhoA (pRhoA), phosphorylated SHP2 (pSHP2), and total SHP2 (tSHP2) antibodies. GAPDH was used as a loading control.

Figure 5. LM11A-31 inhibits cisplatin-induced neurodegeneration

Mouse cortical neurons grown for 5 days *in vitro* (DIV) were treated for 24 hours with cisplatin (8 $\mu\text{g}/\mu\text{L}$), at 6 DIV with LM11A-31 for 24 hours (100nM), or at 7 DIV with calpeptin for 30 minutes (1 unit/mL). (A) Neurons were labeled with FITC-phalloidin (green); nuclei were labeled with Hoechst (inset, blue). Quantification of branch number (B), sprout number (C), branch length (D) and pRhoA pixel intensity (E). Values represent mean $\pm$ S.D. *, relative to saline; Δ , relative to cisplatin; Φ , relative to calpeptin. n= 3 representative neurons/ treatment group.

Figure 6. LM11A-31 inhibits RhoA activity stimulated by cisplatin and calpeptin

Mouse cortical neurons grown for 5 days *in vitro* (DIV) were treated for 24 hours with cisplatin (8 μ g/mL), at 6 DIV with LM11A-31 for 24 hours (100nM), or at 7 DIV with calpeptin for 30 minutes (1 unit/mL). (A) Neurons were labeled with total RhoA (top panel) and phosphorylated RhoA (lower panel). Quantification of total RhoA pixel intensity (B), phosphorylated RhoA pixel intensity (C) and a ratio of the pixel intensity of phosphorylated RhoA to that of total RhoA (D) were calculated. $n=3$ representative neurons/ treatment group. Western blot analysis of phosphorylated RhoA (E) and phosphorylated SHP2 (F) were also performed and quantified. Values represent mean $\pm$ S.D. *, relative to saline; Φ , relative to cisplatin; Δ , relative to calpeptin.

Figure 7. Proposed mechanism of cisplatin-induced peripheral neuropathy

Cisplatin-induced cellular stress (DNA and/or mitochondrial) may result in the activation of both the p75^{NTR} receptor and SHP2 tyrosine phosphatase, through which RhoA can be activated, and resulting in neuronal retraction and repulsion can result.