Published in final edited form as:

Neurotoxicology. 2010 March ; 31(2): 188–194. doi:10.1016/j.neuro.2009.12.010.

Rho Kinase Inhibitor Y-27632 Facilitates Recovery from Experimental Peripheral Neuropathy Induced by Anti-Cancer Drug Cisplatin

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Abstract

Chemotherapy drugs have neurotoxicity associated with treatment, which can become a dose-limiting problem when clinical presentation is severe. However, there is no effective therapy to circumvent the neurotoxicity of anti-cancer drug treatment. In this study, we utilized a newly designed mouse model of cisplatin-induced peripheral neuropathy to determine both the severity of neurotoxicity induced by drug treatment and the effectiveness of the Rho kinase inhibitor Y-27632 in post-treatment recovery. Sensory nerve conduction studies revealed a significant increase in mean distal (peak) latency with cisplatin treatment, indicating a deterioration of sensory nerve function. Also, hind paw touch sensitivity decreased steadily with increasing cumulative dose of cisplatin. Histological and immunohistochemical analyses of the sural nerve using neuronal marker protein gene product 9.5 (PGP 9.5) demonstrated abnormal nerve fiber morphology in cisplatin-treated mice. Remarkably, post-treatment with Y-27632 improved the sural nerve distal (peak) latency and sensory threshold to return to pre-treatment levels. Sural nerve histology worsened in the absence of Y-27632 during recovery. These studies suggest that Rho kinase inhibitor Y-27632 can initiate regeneration of damaged nerves following cisplatin treatment.

Keywords

cisplatin; peripheral neuropathy; regeneration; Rho GTPases; Y-27632

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1. INTRODUCTION

Chemotherapy drugs have been at the frontlines of the war against cancer for the past six decades. Platinum compounds were among the original drugs discovered to hinder the proliferation of cancer cells and proved to be extremely effective in causing remission of germ cell derivative cancers [1]. Cisplatin was originally found to have anti-cancer properties from experiments using tumor bearing mice in 1968 [2]. The results from the preliminary studies were remarkably convincing and patients were treated with cisplatin for the first time in 1971. Cisplatin received FDA approval for cancer treatment shortly thereafter [3]. Before cisplatin was available for use, metastatic testicular cancer had a mortality rate of 95%. The advent of cisplatin-based regimens resulted in a 60% cure rate for this disease [4]. However, cisplatin exhibits a high level of toxicity with treatment, with a majority of patients exhibiting symptoms of neuropathy such as numbness, tingling, or burning in the extremities [5]. At higher doses, almost all patients complain of neurological problems, including cognitive impairment.

Cisplatin has been demonstrated to have an increased affinity for DNA in dorsal root ganglion neurons [6]. An increase in cisplatin level inside the cell generally correlates with the probability of apoptosis [7]. However, it is debatable whether or not apoptosis is the sole mechanism involved in neuropathy. This mechanism alone does not necessarily account for the difference in cisplatin toxicity between patients, with some patients achieving a full recovery while others have years of persistent neuropathic issues. Dorsal root ganglion neurons do not proliferate, and thus an apoptosis only mode of action is less likely.

In other models of nerve tissue injury such as traumatic brain injury, spinal cord injury, and sciatic nerve crush, the Rho small GTPase is activated [8-13]. Inflammatory stimuli upregulate Rho signaling and may be the reason for unsuccessful nerve regeneration following an injury [14]. Multiple factors converge on the Rho GTPases to modulate actin cytoskeleton dynamics and govern the plasticity of neurons. Our recent studies have shown an increase in Rho activity corresponding with cisplatin treatment of primary rat cortical neurons in culture, and a decrease in that activity when a p75^{NTR} receptor ligand mimetic is used to inhibit Rho activity [15]. Furthermore, neuronal recovery was facilitated when cultured neurons were treated with Y-27632, a selective inhibitor for p160^{ROCK}/Rho kinase. These findings were further validated using both a primary dorsal horn/dorsal root ganglion model and a PC12 neuron-like cell model [15]. These studies provided evidence of a potential role of Rho pathway inhibition in neuroprotection from cisplatin-induced injury.

To investigate the hypothesis that Rho signaling inhibition facilitates the recovery from anti-cancer drug-induced neurotoxicity *in vivo*, we established a mouse model of cisplatin-induced peripheral neuropathy to ascertain the efficacy of Rho kinase inhibitor Y-27632. Neuropathy was tested using sural nerve conduction studies, sensory threshold analysis, and sural nerve histological analysis. We identified a significant increase in mean distal (peak) latency with cisplatin treatment, indicating a deterioration of sensory nerve function. Also, hind paw touch sensitivity decreased steadily with increasing cumulative dose of cisplatin. Histological and immunohistochemical analyses of the sural nerve using PGP 9.5 demonstrated abnormal nerve fiber morphology in cisplatin-treated mice. Remarkably, post-treatment with Y-27632 improved the sural nerve distal (peak) latency and sensory threshold to pre-treatment levels, but sural nerve histology worsened in the absence of Y-27632 during recovery. These studies highlight the potential of Rho kinase inhibitors to initiate regeneration of damaged nerves following cisplatin treatment.

2. MATERIAL AND METHODS

2.1 Pharmacological treatments

C57BL6 mice, 3-6 months of age, were treated with either 6µg/g body weight cisplatin (Sigma Co, St. Louis, MO) or 200µl of 0.9% saline solution (experimental control group). Each drug dose was administered via an intraperitoneal injection with animals receiving one dose every 3 weeks for a period of 15 weeks. At the cessation of treatment, each animal was allowed to recover for 30 days. During the recovery period the animals were treated with either Rho kinase inhibitor Y-27632 (Calbiochem, La Jolla, CA), or saline. Y-27632 was applied at 30µg/g every other day for the 30 days of the recovery period. All animal experiments and testing were approved by the East Carolina University Animal Care and Use Committee.

2.2 Sural nerve conductance

The recording of sural nerve conductance was performed essentially as described [16]. Briefly, each mouse was anesthetized using 2% isoflurane administered using a vaporizer and nose cone. The hind limb of the mouse was shaved to allow for better visualization and contact. A temperature controller with rectal temperature probe and heating pad (FHC, Bowdoinham, ME) was used to maintain a temperature of 37°±1 for all electrophysiological recordings. Stainless steel bare needles (Ambu, Glen Burnie, MD) were placed subdermally lateral to the knee for stimulation. Stainless steel 0.008 inch diameter wire attached to micrograbbers (Biopac Systems, Goleta, CA) were used for the active electrode and reference electrode with the active being 1.5cm from the cathode electrode. An unshielded Ag-AgCl electrode with contact posts (Biopac Systems, Goleta, CA) was placed under the abdominal area of the animal to achieve grounding [16]. Mean distal latency and mean amplitude were obtained and recorded using a TECA Synergy™ electrodiagnostic machine. The sural nerve conductance of the mice was obtained for each hind limb before and after treatment ± cisplatin and at the end of the 30-day post-treatment recovery period ± Y-27632.

2.3 Hind paw sensory threshold testing

Animals were subjected to hind paw sensory threshold testing every week for the entire 19 week protocol. The animals were placed on an elevated screen in a clear plastic barrier to allow visualization from all angles. Mice were allowed to become acclimated to the testing environment for 15 minutes before the commencement of testing. A midrange von Frey/Semmes-Weinstein monofilament (Stoelting Co., Wood Dale, IL) was applied at the point of bending to the plantar surface of each hind paw. The touch response of each hind paw was recorded. Monofilament testing proceeded from the smallest diameter until the monofilament applied elicited a touch response. The diameter of the last monofilament to cause a reaction was recorded and used to calculate the force required to produce a reaction in the tested animal. A response was counted as positive if the mouse responded a minimum of 3 out of 5 filament applications.

2.4 Histological methods

Animals were deeply anesthetized using 3% isoflurane vapor and the chest cavity was subsequently exposed. A perfusion needle was inserted into the base of the heart and the animal was perfused with 30 ml of ice-cold sodium nitrite. Immediately, the mouse was perfused with freshly prepared, chilled 4% paraformaldehyde for rapid fixation of nervous tissue. Sciatic (with sural) nerves were rapidly removed and placed in 4% paraformaldehyde/30% sucrose in PBS solution overnight at 4°C for fixation and cryoprotection. The tissues were embedded in OCT and rapidly frozen using liquid nitrogen chilled isopentane. Sections were acquired from the proximal sural nerve at the point of bifurcation from the sciatic nerve. Multiple 10µm frozen sections were affixed to Superfrost (Fisher Scientific, Pittsburgh, PA) slides and

immunostained with rabbit anti-PGP 9.5 (Chemicon, Temecula, CA) using the Vectastain ABC method (Vector Labs, Southfield, MI) with luxol fast blue counterstaining. ABC staining was performed according to the manufacturer's suggestions. Sections were dehydrated using 95% ethanol and incubated in luxol fast blue stain overnight at 37°C for counterstaining. The sections were washed with distilled water and 70% ethanol before mounting on a coverslip for light microscopy. The person not involved in physiological experiments analyzed the histological sections of the mouse sural nerve.

2.4 Statistical analysis

Data obtained from each limb of each mouse was used to compare the results of drug treatments. The sensory thresholds and sensory nerve action potentials were compared using *t*-test analysis. Paired *t*-tests did not demonstrate a significant difference between limbs or between sexes.

3. RESULTS

3.1 Mean distal latency is significantly increased after cisplatin treatment

Adult C57BL6 mice were treated with 5 successive doses of cisplatin at 6µg/g body weight (experimental group) or saline (experimental control group). The doses were chosen because they reflected clinically relevant amount and have been widely used in other animal studies [15,29, and 31]. The doses were given in 3-week intervals and animals were carefully monitored for changes during the experimental treatment period. Cisplatin-treated animals exhibited some clinical signs of chemotherapy treatment, such as partial hair loss and intermittent weight loss. Mice did not display signs of pain or discomfort associated with the treatment, and all procedures were performed in accordance with the Institutional Animal Care and Use Committee.

Sural nerve conductance studies were performed on all animals to test for sensory nerve integrity. The sural nerve is a purely sensory branch of the sciatic nerve which is responsible for the transduction of sensory information from the hind limb. Stimulation electrodes were placed near the bifurcation of the sural nerve from the sciatic nerve, with active and reference electrodes placed at fixed distances at the distal limb. Near nerve stimulation with distal recording of the sensory nerve action potential (SNAP) resulted in clear and consistent recordings. Normal untreated mice (n=13; 7 males, 6 females) were used to determine the baseline values for this nerve conduction method using this mouse strain [16]. The baseline SNAPs for this strain had a mean distal latency of 1.9 ms with a peak-to-peak amplitude of 24µV.

SNAPs obtained from cisplatin treated mice (Fig 1B) were quite different from those acquired from saline treated mice (Fig 1A). Significantly higher mean distal latencies were recorded from cisplatin-treated mice (n=10; 5 male, 5 female) when compared with saline treated controls (n=9; 7 male, 2 female). In fact, ~30 percent of the animals treated with 5 cumulative doses of cisplatin had no detectable SNAP. In this circumstance, the stimulus was increased in an attempt to obtain a sensory reading, however the motor component of the nearby mixed sciatic nerve buried the sensory SNAP. Compound motor action potentials (CMAP) were easily distinguishable from SNAPs because of the high amplitudes of the potential which are measured in millivolts instead of microvolts. The cisplatin-treated animals, with a diminished but detectable nerve conductance, had a mean distal latency of 2.8 ms and the saline treated group was 1.9 ms (Fig 1C). The action potential amplitude of cisplatin treated animals was 18.33 µV, slightly lower than the saline group at 24.6 µV (Fig 1D). The results of the sural nerve conduction studies are consistent with the sensory polyneuropathy found in humans receiving cisplatin therapy.

3.2 Hind paw touch perception is diminished following cisplatin treatment

Von Frey/Semmes-Weinstein monofilament testing serves to investigate the involvement of touch perception of the hind paw [17], which is highly indicative of chemotherapy induced peripheral neuropathy. In our studies, we examined the hind paw touch response of saline and cisplatin-treated animals. The hind paw of each animal was tested using the application of monofilaments with a wide range of diameters. The monofilament diameter correlates with the amount of force applied to the hind paw. The touch sensory threshold force was recorded and used to determine the loss of touch sensation due to neurotoxicity.

Mice in the saline-treated group did not have any significant difference in touch perception while being tested over the 15-week course of treatment (Fig 2). The force applied was within the normal range of touch perception as determined using Semmes-Weinstein monofilament application on untreated normal mice of the same strain. However, cisplatin-treated mice displayed a progressive loss of touch sensation with increasing cumulative dose of cisplatin (Fig 2). These mice required the application of more force on the plantar surface of the hind paw in order to elicit a touch response to the monofilaments. After the fourth round of cisplatin treatment, the mice had a decline in touch sensation that is deemed to be detrimental by clinical standards. Mice no longer had protective touch sensation by the cessation of cisplatin treatment.

3.3 Sural nerve histology reveals a loss of axonal integrity in cisplatin-treated mice

After 15 weeks of drug treatment, the mice were sacrificed and nervous tissue samples were harvested for histological analysis and comparison. Nerve tissues were frozen, fixed and 10 µm cryosections were prepared for immunohistochemistry for protein gene product 9.5 (PGP 9.5) expression with either luxol fast blue or H&E counterstaining. PGP 9.5 is a marker for ubiquitin carboxy-terminal hydrolase L1 which is enriched in neuronal axons and therefore frequently used as a pan-axonal marker [18,19]. Luxol fast blue counterstaining is a technique used to identify myelinated nerve fibers in nervous tissue sections [20].

Sural nerve PGP 9.5 immunostaining revealed dramatic differences between the saline (Fig 3A and B) and cisplatin-treated nerve sections (Fig 3C and D). Nerve fibers in saline-treated animals demonstrated strong PGP 9.5 staining (Fig 3B, asterisk). Analyses of multiple tissue sections revealed that the nerve fibers had a rounded morphology with a substantial myelin covering (Fig 3B, arrows) typical of peripheral sensory nerve fibers. However, in cisplatin-treated samples, some fibers exhibited an abnormal flattened appearance (Fig 3D, arrows), and the luxol fast blue myelin staining was reduced (Fig 3C and D). This immunohistochemical data indicated the loss of nerve integrity and myelination within the sural nerve which was consistent with the reduction of sural nerve conduction and increase in sensory (touch) threshold.

3.4 Y-27632 improves the mean distal latency of cisplatin-treated mice

Our previous work has implicated a role for the Rho GTPase pathway in providing neuroprotection from cisplatin-related neurotoxicity in culture [15]. We demonstrated that inhibition of the Rho pathway with Rho kinase inhibitor Y-27632 facilitates the recovery from cisplatin-induced neurotoxicity *in vitro*. To test the effects of Y-27632 using this *in vivo* model, we divided the cisplatin-treated animals into two subgroups. One group was subsequently treated with saline (n=5; 2 male, 3 female) and the other group received Y-27632 (n=5; 3 male, 2 female). Drugs were applied for one month following the end of cisplatin treatment. Recovery from cisplatin-induced neuropathy was measured by comparison of the previously described indicators, including sural nerve conduction, sensory threshold, and peripheral nerve histology.

Mice recovering from cisplatin treatment with saline administration (without Y-27632) demonstrated some recovery of sensory nerve conductance (Fig 4A). However, the mean distal

latency at 3.25 ms (Fig 4C) was still significantly outside of the normal range (Fig 1D, 1.9 ms). On the other hand, animals with severely impaired SNAPs before the recovery period were capable of producing normal action potentials with normal mean distal latencies after recovery treatment with Y-27632 (Fig 4B). The group of mice treated with Y-27632 was found to have a sural nerve mean distal latency of 2.035 ms, which is within the normal range for this mouse strain (Fig 1A and D). These data indicated that Rho pathway inhibition by Y-27632 facilitates rapid recovery of peripheral nerves following cisplatin-induced injury.

3.5 Y-27632 improves touch sensory perception following cisplatin-induced neuropathy

The sensory threshold (touch sensation) analysis indicated a progressive recovery in both the saline and Y-27632 treated neuropathic mice. The group of mice recovering without the Rho kinase inhibitor Y-27632 showed an improvement, but only a minimal recovery of touch sensitivity occurred (Fig 5). The threshold force remained indicative of touch sensation loss. Similar to the results of the nerve conduction studies, while cisplatin-treated mice were less sensitive to touch stimuli, the sensitivity was returned to the baseline level after treatment with Y-27632 (Fig 5). This indicated a successful functional recovery from cisplatin injury in the presence of Y-27632. In all animals, the sensory threshold analysis was highly correlated to the mean distal latency, and therefore provided for blinded analysis of functional recovery.

3.6 Improved morphological recovery of the sural nerve with Y-27632

We performed histological comparisons of the sural nerve to examine the effects of Y-27632 on the recovery of the anatomical nerve structure. In the group of mice with only saline-assisted recovery (Fig 6A and B), nerve fibers still appeared flattened (Fig 6B, arrows) and myelin staining was further reduced. The demyelination in this group was increased when compared to that of animals immediately following cisplatin treatment. This data indicates the prolonged effects of cisplatin treatment beyond the end of treatment.

On the other hand, Y-27632 was effective in maintaining and improving the integrity of distal nerve fibers in the sciatic nerve (Fig 6C and D). The nerve fibers of Y-27632-treated animals demonstrated intense PGP 9.5 staining with stronger myelin staining when compared to that of animals immediately following cisplatin treatment (Fig 6C and D). Furthermore, some of the fibers appeared to regain a normal rounded morphology (Fig 6D, arrows).

4. DISCUSSION

The current study investigated a new animal model of cisplatin-induced peripheral neuropathy and demonstrated the efficacy of selective Rho kinase inhibitor Y-27632 in enhancing recovery. Mice received a clinically relevant dose of the anti-cancer drug cisplatin, which displays neuropathy as one of the major side effects of clinical treatment. The mice exhibited a slowing of sural nerve conductance as evidenced by prolonged mean distal (peak) latency recordings. The peak-to-peak amplitude was only slightly reduced. Although the mean amplitude was not significantly reduced, the presence of SNAPs was not obtainable in 30% of the mice receiving cisplatin. Therefore, the overall nerve conductance of the sural nerve was impaired in cisplatin-treated mice.

Cisplatin-treated mice also showed a functional sensory deficit in hind paw touch sensation. Sensory threshold analysis using von Frey/Semmes-Weinstein monofilaments was quite sensitive in gauging neuropathy as evidenced from the nerve conduction studies. The threshold force needed to elicit a touch response was increased with each successive dose of cisplatin. Sural nerve biopsy revealed a narrowing of nerve fibers, with a decrease in myelin stain density. More importantly, the effects of cisplatin treatment on SNAPs and sensory threshold were returned to normal after Y-27632 treatment. Sciatic nerve fiber degeneration was more

advanced in the saline post-treatment subgroup when compared to that of the Y-27632 treatment group. These data provide compelling evidence for the efficacy of Rho kinase inhibition in the recovery from cisplatin-induced peripheral neuropathy.

Some studies have suggested the neurotoxicity associated with cisplatin is correlative with drug-induced apoptosis [21]. However, our previous studies using primary neuronal cultures demonstrated that the early signs of action of cisplatin on neurons are neurite retraction [15]. Rho GTPases signaling are critically involved in neurite outgrowth, branching, and regeneration [12,27-30]. Our data further suggest that cisplatin effects are the result of an increase in Rho GTPase activity, which commences with the degeneration of established processes. This neurodegeneration is likely initiated from an inflammatory response induced by cisplatin DNA binding [22].

RhoA has been determined to be essential for the activation of NF- κ B following UV radiation and doxorubicin treatment. The DNA damage induced by these processes has been known to induce an increase in NF- κ B and cytokine responses for decades, however the relationship with RhoA has only been confirmed in recent years [23]. In cisplatin-induced peripheral neuropathy, cisplatin binds to the DNA of dorsal root ganglion (DRG) neurons which may trigger the induction of inflammatory pathways. Current research is exploring the increase in cytokine levels during cancer treatment. Several cytokines are elevated, including TNF α , which is capable of activating RhoA GTPase [24]. This activation may produce instability of the distal axon cytoskeleton leading to nerve degeneration.

The complexity of cisplatin cytotoxicity is widely acknowledged. Cisplatin treatment results in DNA damage signal transduction as well as induction of apoptotic and necrotic pathways [25]. In addition to possible simultaneous activation of multiple pathways, there is also cross talk and cooperation between them [26]. Therefore, the future success in combating chemotherapy-induced peripheral neuropathy depends on the definitive resolution of the mechanisms for neurotoxicity. The present studies suggest that the activation of the Rho GTPases may be involved in the resultant neuropathy of cisplatin, and the inhibition of this pathway is a promising target for intervention. Future studies are necessary to definitively relate the activation of RhoA with neuronal injury following chemotherapy treatment *in vivo*. Although this link has yet to be fully established in human, we have established an animal model and proven its efficacy in the investigation of the neuroprotective effects of Rho pathway inhibition in chemotherapy-related neurotoxicity. The clinical use of Rho signaling inhibitors may be useful for the prevention of the long-term neurological deficits following chemotherapy treatment.

Acknowledgments

The authors wish to thank Melissa Clark and Joani Zary for technical assistance and Dr. Teresa Lever for helpful discussions. This study was supported in part by grants from National Institute on Aging AG026630 and National Cancer Institute CA111891 (Q.L.).

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Figure 1. Sensory nerve action potentials (SNAPs) in cisplatin-treated mice

(A) Representation of sural nerve SNAPs from saline-treated mice (n=9) demonstrate a normal morphology. (B) SNAP morphology of cisplatin-treated mice (n=11) is altered when compared to saline-treatment values. Note: after final cisplatin treatments, three mice had no detectable sensory conductance. Here is the example of sural nerve SNAP from one mouse that showed recordable waveform. The gain is 20 microvolts (y axis) with a sweep of 10 milliseconds (x axis). Waveform marker representations: 1= onset latency, 2 = peak latency, 3 = second peak, and 4 = baseline. (C) Cisplatin-treated mice have a significantly increased mean distal latency (peak latency) when compared to the saline only treatment group. (D) The action potential amplitude of cisplatin-treated mice SNAP is decreased, but not statistically significant. $p < 0.001$ ***.

Figure 2. Touch perception of cisplatin-treated mice progressively declines with increasing cumulative dose

Each animal was tested for mechanical (touch) stimulus perception using Von Frey/Semmes-Weinstein monofilaments. The results indicate a decrease in stimulus recognition with increasing dose of cisplatin, while saline-treated animals maintain a baseline level of touch perception. Values represent mean $\pm$ SEM. $p < 0.01^{**}$.

Figure 3. Histological analysis of sural nerve cross sections indicates nerve degeneration in cisplatin-treated mice

A. Protein gene product 9.5 (PGP 9.5) immunostaining was used to visualize nerve fibers (brown to black) in sciatic samples from both saline- (a, asterisk) and cisplatin (b, arrows)-treated animals. Luxol fast blue counterstaining was used to recognize myelin sheaths (a, arrows). Examples of cisplatin tissue samples demonstrated a narrowing of axons (b, arrows) when compared to saline controls. Scale bar: 50µm. B. Quantification of % of myelin covered nerve fibers in saline- or cisplatin-treated mouse sural nerves. Values represent mean ± SEM. $p < 0.05^*$.

Figure 4. Sural nerve SNAPs in mice recovering from cisplatin-induced peripheral neuropathy
Cisplatin-treated animals are allowed to recover for a one-month period while receiving either saline (n=5) or Y-27632 (n=5). (A) Animals receiving saline during recovery maintain an altered SNAP morphology a month after cessation of cisplatin treatment. (B) Y-27632 treatment during recovery returns SNAP morphology to that detected in normal animals. The gain is 20 microvolts with a sweep of 10 milliseconds. Waveform marker representations: 1 = onset latency, 2 = peak latency, 3 = second peak, and 4 = baseline. (C). Animals receiving Y-27632 treatment were able to demonstrate normal mean distal latencies after the recovery period. Latency did not improve for the group treated with saline during recovery. $P < 0.05^*$

Figure 5. Y-27632-treated mice demonstrate recovery from cisplatin-induced touch sensation deficits

Cisplatin-treated mice underwent a one-month recovery period following drug treatment. During the recovery period, the animals were randomly selected to receive either saline or Y-27632. Mice treated with Y-27632 demonstrated an increase in functional recovery of touch sensation when compared to saline recovery group. Values represent mean $\pm$ SEM. $P < 0.01$ **

Figure 6. Histological analysis of sural nerve cross sections from mice recovering from cisplatin-induced peripheral neuropathy

PGP 9.5 immunostaining of nerve sections revealed nerve fiber bundles (brown-black) with luxol fast blue staining of myelin sheaths (blue). A. Examples of mice treated with saline (a) have a reduction in myelin staining and nerve fibers remain flattened (arrows) whereas Y-27632-treated animal nerve sections (b) have a more robust presence of myelin (arrows). Some normalization of the rounded nerve fiber bundle morphology is clearly visible (b, arrows). Scale bar: 50 μ m. B. Quantification of % of myelin covered nerve fibers in saline- or cisplatin-treated mouse sural nerves during recovery. Values represent mean $\pm$ SEM. $p < 0.05$.*