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Targeting Heat Shock Protein 70 to Ameliorate c-Jun Expression and Improve Demyelinating Neuropathy

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Abstract

Increased expression of the c-jun transcription factor occurs in a variety of human neuropathies and is critical in promoting Schwann cell (SC) dedifferentiation and loss of the myelinated phenotype. Using cell culture models, we previously identified KU-32 as a novobiocin-based C-terminal heat shock protein 90 (Hsp90) inhibitor that decreased c-jun expression and the extent of demyelination. Additional chemical optimization has yielded KU-596 as a neuroprotective novologue whose mechanistic efficacy to improve a metabolic neuropathy requires the expression of Hsp70. The current study examined whether KU-596 therapy could decrease c-jun expression and improve motor function in an inducible transgenic model of a SC-specific demyelinating neuropathy (MPZ-Raf mice). Treating MPZ-Raf mice with tamoxifen activates the MAPK kinase pathway, increases c-jun expression and produces a profound demyelinating neuropathy characterized by a loss of motor function and paraparesis. KU-596 therapy did not interfere with MAPK activation but reduced c-jun expression, significantly improved motor performance, and ameliorated the extent of peripheral nerve demyelination in both prevention and intervention studies. Hsp70 was necessary for the drug's neuroprotective efficacy since MPZ-Raf × Hsp70 knockout mice did not respond to KU-596 therapy. Collectively, our data indicate that modulating Hsp70 may provide a novel therapeutic approach to attenuate SC c-jun expression and ameliorate the onset of certain demyelinating neuropathies in humans.

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Notes

The authors declare the following competing financial interest(s): B.S.J.B. and R.T.D. are named inventors on US and foreign patent applications claiming KU-596 and related compounds, which are among a series of patents and patent applications licensed to Reata Pharmaceuticals by the University of Kansas.

Graphical Abstract

Keywords

C-terminal Hsp90 inhibitors; demyelination; molecular chaperones; neuroprotection; paraparesis; Schwann cells

INTRODUCTION

Peripheral neuropathies are generally classified as axonal or demyelinating and manifest from diverse etiologic backgrounds, e.g., genetic, metabolic, autoimmune, drug, or trauma-induced. Demyelinating neuropathies are typically characterized by damaged myelin but intact axons. At the molecular level, recent evidence suggests that the c-jun transcription factor is a critical node in regulating demyelination.¹ Interestingly, upregulation of c-jun is reported in human axonal and demyelinating neuropathies including Charcot–Marie–Tooth disease (CMT) Type 1A (CMT1A), X-linked CMT (CMT1X,) and chronic inflammatory demyelinating polyneuropathy.^{2,3} However, it remains unclear if targeting c-jun may provide a viable approach to ameliorate certain neuropathies.

c-Jun is highly expressed in immature Schwann cells (SC) and is downregulated as SC begin to myelinate.⁴ Although c-jun is minimally expressed in healthy nerves, it is rapidly upregulated in pathological or injured nerves. During nerve injury, loss of axonal contact causes myelinated SC to arrest myelination and dedifferentiate into an immature phenotype.¹ c-Jun is required for SC dedifferentiation since its SC-specific deletion results in a marked delay in the rate of myelin sheath degradation.¹ However, despite the role of c-jun-N-terminal kinase (JNK) in regulating the transcriptional activity of c-jun by phosphorylation, c-jun-mediated demyelination appears to be independent of this pathway.^{1,5} Thus, pharmacological approaches that antagonize c-jun expression may yield a novel strategy to treat certain human neuropathies. To this end, we have found that modulating molecular chaperones can antagonize the expression of c-jun and subsequent demyelination in SC-DRG (dorsal root ganglia) cocultures.⁶

Molecular chaperones, such as heat shock proteins 70 (Hsp70) and Hsp90, function in the folding of nascent polypeptides, refolding of misfolded proteins and the clearing of damaged proteins or protein aggregates.^{7,8} Hsp90 contains N- and C-terminal nucleotide-binding domains that are joined by a middle domain, which binds cochaperones, client proteins and the transcription factor, heat shock factor 1 (HSF1).⁹ The C-terminal domain is essential for forming a functional Hsp90 homodimer and the N-terminal ATPase is required for the protein's chaperone activity.^{9,10} Through its intrinsic ATPase activity and interaction with cochaperones, isomerases, and immunophilins, Hsp90 directs the folding of "client" proteins into their biologically active conformations.^{11–13} Inhibiting this ATPase activity can increase client protein degradation and may be useful in treating certain malignancies. However, Hsp90 also serves another important biologic role since it is a direct regulator of the cellular heat shock response (HSR) by binding to HSF1 and suppressing its transactivating capacity.¹⁴ Upon exposure to proteotoxic stress or binding of small molecules, conformational changes in Hsp90 disrupt its interaction with HSF1 and lead to the transcriptional induction of cytoprotective proteins, such as Hsp70.

Modulating Hsp70 expression is emerging as an attractive target to improve neurodegenerative diseases associated with protein aggregation. In this respect, N-terminal Hsp90 inhibitors have been reported to decrease tau aggregation in Alzheimer's disease,^{15,16} huntingtin protein in motor neurons,¹⁷ and peripheral myelin protein 22 aggregates in CMT1A mice.¹⁸ However, many N-terminal inhibitors are unable to dissociate client protein degradation from induction of the HSR. This limitation can increase the degradation of Hsp90 client proteins necessary for normal cell homeostasis and antagonize the neuroprotective phenotype. Therefore, an effective Hsp90 modulator for treating neurodegeneration requires a drug with a sufficient therapeutic window that can upregulate Hsp70 but induce minimal client protein degradation to antagonize the protective HSR. To this end, we have identified a series of compounds called novologues that bind to the C-terminal domain of Hsp90 and induce Hsp70 at concentrations ~500-fold lower than those that promote client protein degradation.^{19,20}

KU-596 (Figure 1A) is a highly bioavailable novologue that has shown efficacy in improving sensory deficits in models of diabetic peripheral neuropathy.²¹ The current study assessed the ability of KU-596, to prevent c-jun-induced demyelination in two transgenic mouse models of a SC-specific demyelinating motor neuropathy.²² These proof-of-concept data suggest that attenuating c-jun induction may provide a novel therapeutic strategy in treating certain demyelinating neuropathies.

RESULTS

KU-596 Decreased TMX-Induced c-jun Expression

To investigate the *in vivo* efficacy of KU-596 to attenuate c-jun-induced demyelination, we utilized MPZ-Raf mice. MPZ-Raf mice express a human Raf kinase gene fused to a modified estrogen receptor ligand binding domain downstream of the myelin protein zero (MPZ) promoter.²² MPZ is expressed specifically in SCs and is an important structural component of peripheral nerve myelin. TMX administration activates the Raf kinase-fusion protein in myelinated SCs and drives downstream activation of the p42/p44 mitogen-

activated protein kinase (MAPK) pathway, which induces the expression of c-jun. MAPK activation and c-jun induction promotes a rapid and robust motor neuropathy characterized by severe demyelination of peripheral nerves.²²

Male and female MPZ-Raf mice were treated with 2 mg of TMX dissolved in corn oil (CO) every other day for 14 days to induce MAPK activation. KU-596 (20 mg/kg) or vehicle (0.1 M Captisol, CAP) was given orally on alternate days starting from day 0 (Figure 1B). The dose of KU-596 was chosen based on prior studies that 20 mg/kg of the drug could improve diabetic peripheral neuropathy.²¹ After 14 days, the mice were euthanized and immunoblot analysis of sciatic nerve lysates was performed. As expected, treating the MPZ-Raf mice with TMX induced the phosphorylation of MAPK (pMAPK) and increased c-jun expression in sciatic nerve (Figure 1C and D). Although KU-596 treatment did not alter the activation of MAPK, it significantly reduced the level of c-jun (Figure 1D). Since KU-596 did not decrease pMAPK, the decline in c-jun expression is downstream of MAPK activation. Importantly, these results were confirmed using cross sections of sciatic nerves stained with antibodies against c-jun and myelin basic protein (MBP) (Figure 1E and F). TMX increased the number of c-jun positive nuclei and this was significantly attenuated by KU-596. Similarly, TMX also decreased MBP immunoreactivity, which was markedly improved by KU-596 treatment. No c-jun positive nuclei were noted in animals that received only CO and KU-596.

KU-596 Improves Demyelination in MPZ-Raf Mice

To determine the effect of KU-596 on myelination profiles, MPZ-RAF mice were treated with TMX and KU-596 using the dosing schedule shown in Figure 1B. Sciatic nerves were fixed with osmium tetroxide, teased in glycerol and examined under a light microscope. Whereas control mice (CO + CAP, CO + KU-596) showed normal myelination with well demarcated nodes of Ranvier, nerves from TMX + CAP treated mice showed large regions denuded of myelin (Figure 2A). Although KU-596 trended toward reducing the length of the demyelinated region, the effect was variable (Figure 2B). To further quantify the effect, a teased fiber was considered damaged if the distance between two myelinated sections was at least 10% greater than the average distance between internodes of CO + CAP treated animals (Figure 2B). By this criterion, the drug significantly decreased the percentage of damaged fibers (Figure 2C). In addition, cross sections of sciatic nerves indicated an increased number of abnormally myelinated fibers in the TMX+CAP group (Figure 3A). KU-596 treatment reduced by 25% the number of abnormally myelinated fibers defined as those with a myelin sheath, exhibiting myelin splitting, having myelin debris, onion bulbs, myelin ovoids or presence of regeneration clusters (Figure 3B). Despite the rapid onset and robust level of demyelination induced by TMX, these data suggest that KU-596 can ameliorate but not prevent the degeneration.

KU-596 Improves Motor Function in MPZ-Raf Mice

Since demyelination mainly affects large caliber motor nerves, we sought to test the effects of TMX and KU-596 on motor function by examining the ability of the mice to stay on a ramping rotarod. Animals were randomly assigned to each of the four treatment groups and given five training trials on the rotarod. MPZ-RAF mice were treated using an alternate day

dosing schedule with day 0 serving as the baseline rotorod performance prior to any treatment. Animals were given 20 mg/kg KU-596 on alternate days beginning at day 0, while TMX was administered every other day beginning at day 1.

As expected,²² TMX + CAP treated mice developed an impaired motor coordination and showed a sharp decrease in the latency to fall beginning at day 10 (after 4 TMX doses) (Figure 4A and B). Compared to mice treated with TMX + CAP mice, animals that received TMX + KU-596 showed a significantly delayed onset of the motor deficit and a preservation of motor function. Indeed, TMX+CAP mice showed an abnormal posture and rear limb positioning with the most severe mice exhibiting paraparesis (Figure 4C, top). In contrast, TMX + KU-596 treated mice had a relatively normal posture (Figure 4C, bottom), the limbs were able to support the body weight and the mice more effectively used all four limbs when ambulating, as discussed below.

Since the rotorod test is a rather gross indication of motor coordination, we employed a force plate actometer (FPA) to quantify locomotor activity with high temporal (10 ms) and spatial (2 mm) resolution.^{23,24} No training of the animal is required since the FPA measures multiple movement parameters as the mouse freely explores the FPA chamber during a 10 min session. Animals were treated with TMX and KU-596 as discussed above and motor function in the FPA was assessed on day 14. Figure 4Di shows representative patterns of movement and overall distance traveled by the mice in the FPA chamber. Compared to control mice, TMX treated mice tended to restrict their movement around the perimeter of the chamber and showed a significant decrease in the total distance traveled from 25.4 to 6.7 m. This is likely due to the extensive paraparesis (Figure 4C) and not a behavioral deficit induced by TMX; WT mice treated with TMX showed normal exploratory behavior (data not shown). In contrast, the overall distance traveled by mice receiving TMX + KU-596 was significantly increased compared to the TMX + CAP group, and approximated the exploratory behavior of the control mice, though it remained slightly decreased.

The FPA also measures ambulation rhythmicity (stride) and force of paw placement during the strides. The panels in Figure 4Diii show representative force–time waveforms of paw placement forces during a straight run in the FPA. The symmetry of the force–time peaks represents the rhythmicity of ambulation during the series of strides while the amplitude of the wave measures the force of paw placement (power output). To quantify power output, it is normalized to body weight (% bw) so that variations in amplitude are independent of this variable.²³ TMX treatment clearly abolished the peak symmetry (rhythmicity of the stride) and peak amplitude (force of the paw hitting the force plate). The panels in Figure 4Div are a magnified view of the force data from one animal per group to highlight the differences in movement. These deficits result from the loss of limb coordination (Figure 4C) and inability of the limbs to sustain the body weight. Changes in force can be quantified following Fourier transformation of the waveforms which produces a power spectra (Figure 4Dii). The peak around 8–9 Hz in control mice represents normal ambulation rhythmicity and integration of the area under the curve (AUC) provides an indication of the force. TMX clearly abolished rhythmicity and significantly decreased the AUC in the power spectra. However, mice treated with KU-596 showed a significant improvement in ambulation rhythmicity and force. Together, these data indicate that decreased c-jun expression and the

improved myelination following KU-596 treatment correlated with the development of a less severe motor neuropathy.

KU-596 Improves a Pre-Existing Motor Deficit in MPZ-Raf Mice

To test the ability of KU-596 to reverse a preexisting motor deficit, rotarod rod performance was assessed every other day for 9 days in a group of 42 MPZ-Raf mice. After each session, the mice were administered TMX every other day for a total of 5 doses over the 9 days (Figure 5A). By day 11, the latency to fall was approximately 75% of the baseline on day 1. After assessing the rotarod latency on day 11, the mice were randomly assigned to one of four groups which received daily doses of 0 ($n = 12$), 0.1, 1, or 10 mg/kg of KU-596 ($n = 10$ each) from days 11 to day 18. This log range was chosen since 0.1 mg/kg shows minimal efficacy in improving diabetic peripheral neuropathy. Performance on the rotarod was assessed every other day from days 13–19 (Figure 5B). The latency to fall in mice receiving 0.1 mg/kg of KU-596 remained significantly impaired compared to the control mice and was indistinguishable from the mice treated with TMX + CAP. Mice receiving 1 or 10 mg/kg KU-596 did show some improvement in the latency to fall since they were not significantly different from the CO control group at days 13, 15, and 19. However, the magnitude of this improvement was not sufficient to be statistically different relative to the TMX + CAP group at any of the time points. Analysis of the latency to fall responses of individual animals indicated a rather wide variability among those treated with either TMX + CAP or TMX + KU-596 (data not shown). Therefore, to minimize the effect of animals that were either poor or good responders, the median fall latency in each group at each time was used to determine if the drug was having a significant effect over the duration of treatment. The median fall latencies for each treatment at days 13–19 were combined (4 time points per group) and analyzed by one-way ANOVA and Scheffe's posthoc test (Figure 5D). This analysis suggests that daily dosing with 1 and 10 mg/kg KU-596 from days 11–18 significantly increased the fall latency over this treatment period.

Hsp70 Is Required for Drug Efficacy

Our previous work indicated that the ability of KU-596 to improve sensory deficits associated with the development of DPN is Hsp70-dependent.^{6,19,21,25} However, demyelination is not a hallmark of the sensory neuropathy that develops in diabetic rodents. Therefore, to determine if the ability of KU-596 to improve the demyelinating motor neuropathy in MPZ-Raf mice is also Hsp70-dependent, MPZ-Raf $\times$ Hsp70 KO mice were generated (Figure 6A). Importantly, deletion of Hsp70 had no effect in altering the expression of the constitutive isoform, Hsc70, in the sciatic nerve.

Deletion of Hsp70 did not affect basal motor coordination since untreated MPZ-Raf $\times$ Hsp70 KO mice (Figure 6B) performed as well as the MPZ-Raf mice (Figures 4A and 5B) on the rotarod. As described for Figure 4A, MPZ-Raf $\times$ Hsp70 KO mice were also given 20 mg/kg KU-596 on alternate days beginning at day 0, while TMX was administered every other day beginning at day 1. Treating the MPZ-Raf $\times$ Hsp70 KO mice with TMX induced a progressive decrease in the latency to fall from the rotarod indicating that Hsp70 is not necessary to develop the motor neuropathy. However, in contrast to the MPZ-Raf mice, 20 mg/kg of KU-596 was unable to improve rotarod performance (Figure 6B). Consistent with

this result, KU-596 also did not improve the distance traveled in the FPA (Figure 6Ci and D) nor ambulation rhythmicity (Figure 6Cii). Lastly, although TMX increased c-jun levels in sciatic nerve (Figure 6E), KU-596 was unable to decrease c-jun as occurred in the MPZ-Raf mice. These data indicate that KU-596 requires Hsp70 to improve the motor neuropathy and link Hsp70 to the decrease in c-jun.

DISCUSSION

c-Jun belongs to the AP-1 transcription factor family and was first discovered as an oncogene.²⁶ Although elevated c-jun is not the cause of all neuropathies, it may be an important culprit. Parkinson et al. were the first to demonstrate that c-jun is a negative regulator of myelination and is required for SC dedifferentiation.¹ Our data suggests that decreasing c-jun expression with KU-596 therapy correlates with a decrease in demyelination and improved motor function in MPZ-Raf mice, a robust model of a rapid onset demyelinating neuropathy. Moreover, drug efficacy seems to require Hsp70 as improved motor function could not be recapitulated in MPZ-Raf $\times$ Hsp70 KO mice.

Hsp70 is mechanistically known to inhibit the JNK pathway and protect neurons from apoptosis.^{27,28} However, the phosphorylation of c-jun by JNK is not necessary for c-jun to promote demyelination.¹ Our published work using an earlier generation C-terminal Hsp90 inhibitor, KU-32, indicated that an increase in the proteasomal clearance of c-jun and phospho-c-jun was linked to protecting against demyelination in SC-sensory neuron cocultures.⁶ Thus, we anticipate that the decrease of c-jun expression is more likely due to increased clearance rather than decreased production, but this remains to be demonstrated in vivo. One possible mechanism for enhancing c-jun clearance via Hsp70 is through the carboxy terminus of heat shock cognate protein 70 (Hsc70) interacting protein (CHIP). CHIP is an Hsp70 interacting E3 ubiquitin ligase that mediates the proteasomal clearance of Hsp70 substrates by binding to a C-terminal EEVD sequence of the chaperone.²⁹ Since Hsp70 can coimmunoprecipitate c-jun, an Hsp70-CHIP complex may be involved in its clearance. However, no reports have linked CHIP as a critical mediator for c-jun degradation. On the other hand, the F-box protein, Fbw7, is known to enhance the clearance of c-jun by promoting its ubiquitination by the SCF ubiquitin E3 ligase complex.^{30,31} Phosphorylation of c-jun at T²³⁹ and S²⁴³ is required for its recognition by Fbw7³² and TMX-treated mice showed an increase in c-jun phosphorylation at these residues (data not shown). Fbw7 is readily detected in sciatic nerve extracts and can also coimmunoprecipitate with Hsp70 from cell extracts (data not shown). Little is known about how c-jun is cleared in SC and these observations suggest that Fbw7 may regulate c-jun function. The role of Fbw7 in regulating c-jun clearance via KU-596 and Hsp70 is currently being examined using mice with a SC-specific conditional deletion of Fbw7.

Although KU-596 therapy could not decrease c-jun levels in the absence of Hsp70, it remains unclear if a decrease in c-jun is critical to mediating the improvement in nerve function or is a consequence of other mechanisms by which Hsp70 may ameliorate the neuropathy, i.e., enhancing mitochondrial bioenergetics or decreasing inflammation.²¹ Moreover, emerging data suggests that c-jun serves a complicated role in SC function. In response to injury or metabolic insults, robust induction of c-jun in myelinated adult SCs is

recognized as essential for promoting dedifferentiation of the myelinated phenotype.¹ For instance, an increase in SC c-jun expression correlates with the extensive demyelination that occurs in a mouse model of human CMT1X.³³ Though high levels of c-jun expression are sufficient to drive SC dedifferentiation, c-jun levels decline after loss of the myelin membrane. Recent data supports that a low level of c-jun expression in injured SCs may be important to produce trophic factors, such as glial derived neurotrophic factor, that can aid the survival of the desheathed sensory neurons.^{34,35} For example, the C3 mouse model of CMT1A shows a decrease in the number of myelinated sensory axons compared to wild type mice.³⁶ However, SC-specific deletion of c-jun in the C3 mouse exacerbated the loss of myelinated sensory axons, suggesting a supportive role of c-jun in axonal survival.³⁶ While pharmacologic modulation of c-jun levels is distinctly different from its genetic deletion, it is translationally important to determine if the drug may interfere with, complement, or supplant any trophic actions of c-jun in supporting desheathed axons in relevant disease models that have a SC-specific deletion of c-jun.

A second caveat of our work is that the rapid onset demyelinating neuropathy shown by the MPZ-Raf mice does not mimic a human disease. Importantly, c-jun is elevated in several inherited neuropathies, such as CMT1A, CMT1B, and CMT1X.^{3,33} The ability of KU-596 to improve motor function in this rapid and robust model of a demyelinating neuropathy suggest it may have clinical potential in these slower onset diseases since muscle weakness and limited mobility are major complaints from afflicted patients.^{37,38} Since induction of Hsp70 by N-terminal Hsp90 inhibitors can clear protein aggregates in models of CMT1A,^{39,40} KU-596 may be beneficial in treating this inherited neuropathy since it has a broader therapeutic window that promotes Hsp70 induction without increasing client protein degradation, which would be neurotoxic. The application of KU-596 in treating CMT1X would be intriguing as well. Since CMT1X is a mixed axonal and demyelinating neuropathy, decreasing c-jun expression may help improve aspects of the demyelinating neuropathy while providing some support for the axonal neuropathy via its documented ability to improve at least sensory neuron mitochondrial bioenergetics. Furthermore, a SC-dependent secondary inflammation that stimulates macrophage-mediated demyelination contributes to the progressive neuropathy in CMT1X.^{41,42} C-C motif chemokine (CCL2) is derived from SCs, and attracts macrophages to infiltrate the endoneurium and directly contribute to myelin degeneration in CMT1X.⁴³ Since c-jun can transcriptionally increase CCL2,⁴⁴ decreasing its levels may abrogate CCL2 production and decrease macrophage-mediated myelin degeneration in CMT1X. Consistent with this premise, transcriptomic analysis found that KU-596 therapy broadly decreased the expression of inflammatory genes and was predicted to decrease CCL2 activation in diabetic dorsal root ganglia.²¹ The potential utility of KU-596 and Hsp70 in improving physiologic read outs of nerve function in mouse models of CMT1X is currently being assessed.

In summary, we show that novologue therapy can attenuate the severe morphologic and physiologic indices of a demyelinating neuropathy that rapidly occurs in the MPZ-Raf mice. Since human demyelinating neuropathies typically show a more gradual development, this bodes well for the drug's translational potential. Combined with our extensive published data on the efficacy of novologues in attenuating a metabolic neuropathy,^{19,21,25,45} these

data provide strong proof-of-principle that the upregulation of endogenous cytoprotective pathways can attenuate neuropathies of distinct etiologies.

METHODS

Corn oil (CO), *p*-phenylenediamine and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Tamoxifen (TMX) was purchased from Cayman Chemical (Ann Arbor, MI). KU-596, *N*-(2-(5-(((3*R*,4*S*,5*R*)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2-yl)oxy)-3'-fluoro-[1,1'-bi-phenyl]-2-yl)ethyl)-acetamide, was synthesized as previously described.²⁰ Compound purity routinely exceeded 95%.

c-Jun antibody was purchased from Cell Signaling Technology (#9165, Danvers, MA). Biotinylated Hsp70 antibody was purchased from Enzo Life Sciences (#ADI-SPA-810B, Farmingdale, NY). β -Actin antibody, GAPDH antibody, goat anti-mouse HRP conjugated secondary antibody, and goat anti-rabbit HRP conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit Alexa Fluor-647 conjugated secondary antibody, streptavidin-HRP, and Diamond prolong antifade mounting solution with 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo-Fisher Scientific Inc. (Grand Island, NY).

Tissue-Tek Optimum cutting temperature (OCT) was purchased from Electron Microscopy Sciences (Hatfield, PA). Captisol (CAP) was purchased from Cydex Pharmaceuticals Inc. (Lenexa, KS)

Animals

Hemizygous MPZ-Raf mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred to homozygosity. Hsp70.1/Hsp70.3 knockout (Hsp70 KO) were originally obtained from the Mutant Mouse Resource and Research Center (Davis, CA) and MPZ-Raf^{+/+} $\times$ Hsp70 KO mice were generated by crossing the strains. The Raf transgene was confirmed using the following primers: forward: GCAGCCCACACTGAGGATA and reverse: TTGATCGAGTAATCCCCAGCAG, 1.1 kb. Hsp70 was detected using forward: GTACACTTTAACTCCCTCC and reverse: CTGCTTCTCTTGTCTTCG, 450 bp, while the Hsp70 KO was verified using forward: ATGGGATCGGCCATTGAACAAG and reverse: ACTCGTCAAGAAGGCGATAGAAGG, 850 bp. The reactions contained 1 $\times$ PCR buffer, 0.5 mM dNTPs, 0.5 μ M of each primer, 200–300 ng of genomic DNA, and 0.2 U of Omni KlenTaq polymerase (DNA Polymerase Technology, St Louis, MO). PCR conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s; 65 °C for 30 s; 72 °C for 30 s; 72 °C for 5 min. All animals were maintained on a 12 h light/dark cycle and given ad libitum access to chow (NIH diet 7005) and water.

Demyelination was induced by intraperitoneal injection of 2 mg of TMX in CO on administration schedules that are indicated in the text. A stock solution of 4 mg/mL KU-596 in 0.1 M Captisol in phosphate-buffered saline (PBS) was prepared and animals were dosed by oral gavage (0.2 mL) with the dose and administration schedules indicated in the text. Control animals received identical doses of CO and 0.1 M Captisol only. All animals were randomly assigned to each treatment group and both male and female mice were used in all

experiments. TMX treated mice showing extensive limb paralysis were monitored closely with veterinary supervision, given moistened food directly on the cage floor and euthanized if they were unable to access food and water or reached a body condition score of 1.⁴⁶ All procedures complied with protocols approved by the Institutional Animal Care and Use Committee and with National Institutes of Health standards and regulations for the care and use of laboratory rodents.

Immunoblot Analysis

Sciatic nerves were homogenized and sonicated in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS, 0.5 mM sodium orthovanadate, 40 mM NaF, 10 mM β -glycerophosphate, and 1× Complete Protease Inhibitors. Lysates were centrifuged at 10 000g for 10 min at 4 °C and protein concentration was determined using the DC (detergent compatible) protein assay (Bio-Rad Laboratories, Inc.) with bovine serum albumin as the standard. 20 μ g of protein was separated by SDS/PAGE and transferred to nitrocellulose membrane for immunoblot analysis. The membranes were blocked for 1 h with 5% nonfat milk in PBS with 0.1% Tween-20 (PBST) and incubated with primary antibodies overnight at 4 °C. Membranes were washed three times with PBST and incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies for 2 h at room temperature. Following three washes with PBST, immunoreactivity was visualized using enhanced chemiluminescence detection kit (GE Healthcare Life Sciences). The films were scanned and the density of each blot was analyzed using ImageJ.

Immunofluorescence Analysis

Sciatic nerves were harvested from MPZ-Raf mice and immersed in Zamboni's fixative overnight at 4°C. Nerves were washed with PBS and cryoprotected in 30% sucrose until the desired dehydration was achieved. Nerves were mounted in OCT and cut at 10 μ m using a cryostat. Cryosections were collected onto charged slides and stored at -80 °C until use. Sections were rehydrated with PBS (3× for 5 min) and permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature. Tissues were incubated overnight at 4 °C with primary antibodies diluted in 10% normal goat serum in PBS. Sections were washed with PBS and incubated with goat anti-rabbit Alexa Fluor-647 conjugated secondary antibody for 2 h at room temperature. Slides were mounted with Diamond prolong antifade with DAPI and imaged on an Olympus/3I Spinning Disk Confocal/TRIF Inverted Microscope. Five random sections were captured using SlideBook 6.4 (Intelligent Imaging Innovations Inc.). The number of c-jun positive profiles per section was quantified using CellProfiler. Individual nuclei with a diameter of 15–120 pixels were identified using Otsu's thresholding method. Identified nuclei with c-jun intensity greater than 0.03 were classified as positive.

Teased Nerve Fibers

Teased nerves were prepared as previously described.⁴⁷ Sciatic nerves were harvested and fixed with 3% glutaraldehyde overnight at 4 °C. Following 3 washes with 0.1 M phosphate buffer, nerves were secondarily fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide in 0.5 M phosphate buffer for 1 h. Nerves were washed with PBS and incubated

with a series of glycerol solutions (33%, 66%, 100%) for 6 h each. Nerves were stained with 0.6% Sudan Black in 70% ethanol for 30 min, washed with 70% ethanol and water and stored in glycerol. Nerves were teased in glycerol on glass slides, allowed to dry and examined under a light microscope.

Nerve Cross Sections

Sciatic nerves were fixed in 2.5% glutaraldehyde in 0.1 M HEPES, pH 7.4 for 24 h at 4 °C. Nerves were then washed with 0.1 M HEPES and secondarily fixed with 2% osmium tetroxide for 2 h. After washing twice with distilled water, nerves were dehydrated with a series of 30%, 50%, 70% ethanol solutions for 10 min each. Nerves were then rinsed twice with 95% ethanol, 100% ethanol, and propylene oxide for 15 min. The nerves were placed in a mixture of 50% propylene oxide and 50% resin for 2 h, transferred into 100% resin, and placed in a vacuum chamber overnight. Nerves were embedded in flat embedding molds (Ted Pella Inc.) with 100% resin and put in an oven overnight at 60 °C. Sections of 1 μ m were obtained using a Leica Ultratome system (Leica Microsystems Inc.). Sections were collected onto glass slides and stained with 2% p-phenylenediamine in 50% ethanol for 10 s on a hot plate at 65 °C. Slides were washed, a coverslip adhered, and the slides examined by light microscopy.

Behavioral Tests

Mice were placed on an accelerating rotarod (4–40 rpm) (Med Associate Inc., Fairfax, VT) for 5 trials each day with a cutoff of 300s per trial. The latency to fall was recorded and the median of the 5 trials was determined. Mice were trained for 5 days before the start of a study and were tested every other day.

As a second assessment of motor function, locomotor activity was quantified with high temporal (10 ms) and spatial (2 mm) resolution using a force-plate actometer (FPA) (Bioanalytical Systems Inc.).^{23,24} Mice were placed in the FPA chamber for a 10 min session as previously described.⁴⁸ Data was analyzed using custom-written Free Pascal software and the distance traveled in the chamber was calculated. The FPA also measures ambulation rhythmicity (stride) and force of paw placement during the strides. The resulting force-time waveform reflects the rhythmicity of ambulation while the amplitude of the wave measures the force of paw placement during the series of strides. The force data is normalized to body weight (%bw) so that variations in amplitude are independent of this variable.²³ Max power and integrated power output were calculated after Fourier transformation of the force-time waveform data using a Hanning data window and Matlab's Signal Processing Toolbox.

Statistical Tests

Data are presented as means $\pm$ standard error of the mean (SEM). After verifying equality of variance between groups, parametric data were assessed using a one-way analysis of variance (ANOVA) and Tukey's post hoc test. Nonparametric data were analyzed using a Kruskal–Wallis and Dunn's test. A *p* value < 0.05 was considered significant, and for clarity only this value is listed even if the significance was lower. All analyses were performed using Systat (v13.1), ProStat (v4.83), or R (v3.4.1) software.

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ABBREVIATIONS

CAP	Captisol
CO	corn oil
DAPI	4,6-diamidino-2-phenylindole
DRG	dorsal root ganglia
FPA	force plate actometer
HSF1	heat shock factor 1
Hsp70	heat shock protein 70
Hsp70 KO	Hsp70 knockout
Hsp90	heat shock protein 90
HSR	heat shock response
JNK	c-jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MPZ	myelin protein zero
SC	Schwann cells
TMX	tamoxifen
WT	wild-type

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Figure 1.

KU-596 decreases c-jun expression in TMX treated MPZ-RAF mice. (A) Structure of KU-596. (B) Dosing regimen of MPZ-RAF mice. Mice were treated with Captisol (CAP) or KU-596 (20 mg/kg) every other day for 14 days starting from day 0. Beginning on day 1, mice received corn oil (CO) or 2 mg of tamoxifen (TMX) every other day for 14 days. (C) Mice were sacrificed on day 15, lysates of the sciatic nerves prepared and pMAPK, total MAPK, c-jun and β -actin levels determined by immunoblot analysis. (D) Quantification of pMAPK ($n = 3$ per group) and c-jun expression (CO + CAP, $n = 9$; CO + KU-596, $n = 8$; TMX + CAP, $n = 15$; TMX + KU-596, $n = 15$). * $p < 0.05$ compared with CO + CAP. # $p < 0.05$ compared with TMX + CAP. (E) Immunostaining of sciatic nerve cross sections of MPZ-RAF mice. Frozen sections were stained for c-jun (green) and MBP (red). (F) Frozen sections were stained for c-jun (green) and nuclei were visualized with DAPI. The number of c-jun positive nuclei were counted and normalized to the total number of nuclei in two sections obtained from three animals per group. * $p < 0.05$ compared with CO + CAP. # $p < 0.05$ compared with TMX + CAP. Scale bar = 20 μ m.

Figure 2.

KU-596 improves myelin integrity. (A) Representative pictures of the effect of TMX and KU-596 treatment on myelination in teased sciatic nerves of MPZ-RAF mice (scale bar = 10 μm). (B) Quantification of the demyelinated length of sciatic nerves in TMX and TMX + KU-596 treated MPZ-Raf mice. Columns represent individual animals and dots represent length of demyelinated regions in individual teased nerves. Bar indicates median. (C) Quantification of percentage of damaged nerves in each group. Nerves with demyelinated distances that were at least 10% greater than the average distance between two internodes in the CO + CAP treated MPZ-RAF mice were classified as damaged. * $p < 0.05$ compared with CO + CAP. # $p < 0.05$ compared with TMX+CAP.

Figure 3.

KU-596 decreased abnormally myelinated fibers. (A) Cross section of sciatic nerves of MPZ-RAF mice. Examples of myelin splitting (arrows) and demyelinated fibers (arrowheads) are indicated. Scale bar, 10 μ m. (B) Quantification of abnormally myelinated nerves from each group. Abnormal myelination was defined as the presence of myelin splitting, myelin debris, onion bulbs, myelin ovoids and regeneration clusters. Each symbol represents the average from one section that was counted twice in separate sessions while blinded to treatment. The number of animals per group was CO + CAP ($n = 4$), CO + KU-596 ($n = 3$), TMX + CO ($n = 6$) and TMX + KU-596 ($n = 5$). Kruskal–Wallis nonparametric test, $*p < 0.05$ compared with CO + CAP, $\#p < 0.05$ compared with TMX + CAP.

Figure 4.

KU-596 improves motor function of TMX treated MPZ-RAF mice. (A) Latency to fall on the rotarod was assessed in five trials and the median was recorded. KU-596 increased time on the rotarod indicating improved motor function ($n = 8-13$). * $p < 0.05$ compared with CO + CAP. # $p < 0.05$ compared with TMX + CAP. (B) Latency to fall of individual mice from each group on days 10, 12, and 14, respectively. (C) Representative pictures of MPZ-Raf mice treated with TMX + CAP (top) or TMX + KU-596 (bottom) at the end of the study. (D) Mice were placed in the FPA for 10 min and the distance traveled (Di) and paw placement forces (Diii) were recorded. Numerical data for distance traveled in meters are shown in the lower margin of Di for the three groups. The second row of panels (Dii) represents the corresponding Fourier transforms of the force-time waveforms shown in Diii. Together, Dii and Diii establish the rhythmic character and power output of individual bursts of locomotion. The fourth row of panels (Div) show enlarged images of force-time waveforms for the indicated animal in each group to more clearly illustrate treatment-related differences in ambulation power and rhythmicity. * $p < 0.05$ versus CO + CAP; # $p < 0.05$ vs TMX + CAP.

Figure 5.

KU-596 improves a pre-existing motor deficit. (A) TMX was given every other day until the latency to fall dropped to about 75% of baseline by day 11. After the rotorod session on day 11, animals were randomly assigned to be treated daily with 0–10 mg/kg KU-596 and the dosing was initiated. (B) Latency to fall of MPZ-RAF mice on the rotorod. Each mouse received 5 trials and the median of latency to fall was recorded. The arrow points to the start of daily KU-596 or vehicle treatment. TMX produced a statistically significant decrease in response latency that was not improved by 0.1 mg KU-596. Response latency in mice treated with 1 or 10 mg/kg KU-596 improved and was not different from control mice. (C) The medians of each treatment at days 13–19 were grouped and analyzed to minimize variability associated with good versus poor responders. One way ANOVA and Scheffe's posthoc test indicated that 1 and 10 mg/kg KU-596 improved motor function over the duration of treatment. * $p < 0.05$ compared with CO + CAP.

Figure 6.

KU-596 improves the motor deficit in an Hsp70 dependent manner. (A) Left panel shows genotype analysis of the MPZ-Raf × Hsp70 KO mice. A, Raf transgene; B, Hsp70 WT; C, Hsp70 KO. The animal in white font was heterozygous for the Hsp70 KO while the animal in red font was homozygous. The right panel shows immunoblot analysis of sciatic nerve from 2 C57Bl/6 mice and four MPZ-Raf × Hsp70 KO mice. The mice lacked Hsp70 expression and this had no effect on altering the expression of Hsc 70. (B) Mice were given 20 mg/kg KU-596 on alternate days beginning at day 0, while TMX was administered every other day beginning at day 1. Mice were trained for 5 days on the accelerating rotarod and tested every other day. Each mouse received 5 trials and the median latency to fall was recorded. KU-596 treatment showed no effect on the latency to fall compared with the TMX +CAP group. (C) Mice were treated with TMX and KU-596 as described above and on day 15, and the total distance traveled (Ci) was quantified (D). (E) Sciatic nerves were analyzed for c-jun expression by immunoblot and equal loading was verified by immunoblotting for β-actin and via staining with Ponceau red.