Ethoxyquin Prevents Chemotherapy-Induced Neurotoxicity via Hsp90 Modulation

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Objective: Peripheral neurotoxicity is a major dose-limiting side effect of many chemotherapeutic drugs. Currently there are no effective disease-modifying therapies for chemotherapy-induced peripheral neuropathies, but these side effects of chemotherapy are potentially ideal targets for development of neuroprotective therapies, because candidate drugs can be co- or preadministered before the injury to peripheral axons takes place.
Methods: We used a phenotypic drug screening approach to identify ethoxyquin as a potential neuroprotective drug and carried out additional biochemical experiments to identify its mechanism of action.
Results: We validated the screening results with ethoxyquin and its derivatives and showed that they prevented paclitaxel-induced peripheral neuropathy without blocking paclitaxel's ability to kill tumor cells. Furthermore, we demonstrated that ethoxyquin acts by modulating the chaperone activity of heat shock protein 90 (Hsp90) and blocking the binding of 2 of its client proteins, ataxin-2 and Sf3b2. Ethoxyquin-induced reduction in levels of both of these proteins resulted in prevention of axonal degeneration caused by paclitaxel.
Interpretation: Ethoxyquin and its novel derivatives as well as other classes of small molecules that act as Hsp90 modulators may offer a new opportunity for development of drugs to prevent chemotherapy-induced axonal degeneration.

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Paclitaxel (PTX) is a chemotherapeutic drug used extensively to treat a variety of solid tumors, including ovarian, breast, and lung cancer.[1–5] However, like many other chemotherapeutic drugs, PTX can cause severe peripheral neuropathy that affects patients in early stages of treatment and results in long-lasting morbidity.[5–7] Chemotherapy-induced peripheral neuropathy greatly impacts patients' quality of life and limits the use of higher doses of the drug.[3–7] Currently, there is no effective therapy to prevent peripheral neuropathy caused by PTX. Here, we report development of a phenotypic drug-screening assay that resulted in identification of ethoxyquin (EQ) and its derivatives as potential neuroprotective compounds that prevent PTX-induced peripheral neuropathy without blocking PTX's ability to kill tumor cells. Furthermore, we identify heat shock protein 90 (Hsp90) as the key molecule mediating the neuroprotective effects of EQ.

Materials and Methods

All experiments involving animals were carried out according to protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee. Information for all of the antibodies, primers, and siRNA constructs used in the study, including source and catalogue numbers, are listed in the Supplementary Material.

Cell Culture and In Vitro Neuroprotection Assays

Cultures of dorsal root ganglion (DRG) neuronal cell line and neuroprotection assays were carried out as previously described.[8] Conditions for culturing the 50B11 DRG neuronal cells and measuring ATP levels were optimized for the 96-well plate

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format. Briefly, 3,500 cells/well in media (Neurobasal medium, 5μg/ml blasticidin, 10% fetal bovine serum [FBS], 0.5mM glutamine, 1 × B-27 supplement, 0.2% glucose) were plated in 96-well plates for 24 hours, then differentiated for another 24 hours with 100μM forskolin (Sigma-Aldrich, St Louis, MO) in a culture medium with reduced serum (0.2%). In the initial screening of the Spectrum library, drugs were dissolved in dimethylsulfoxide (DMSO) and diluted to the final concentration of 10μM in the culture medium. PTX (100nM), capsaicin (50μM), or zalcitabine (10μM) was used to induce neurotoxicity. Compounds from the Spectrum library were added to the cells with 1 of the toxic compounds. Twenty-four hours later, cellular ATP levels were measured using ViaLight Plus kit (Cambrex, East Rutherford, NJ) according to the manufacturer's protocol on LMax (Molecular Devices, Sunnyvale, CA).

The cells where only vehicles were added served as positive controls, and the toxic drugs (PTX, capsaicin, and zalcitabine) were the negative controls. Each plate contained both positive and negative controls, and the percentage neuroprotection was calculated based on those values for each Spectrum library compound in the plate that it was tested. In dose–response experiments, the concentrations of EQ derivatives and the initial set of hit compounds that provided >50% neuroprotection were varied.

To validate the effect of EQ on actual neurite degeneration induced by PTX, measurements of axonal lengths were done as previously published.[9] Briefly, DRGs were harvested from embryonic day 14.5 rats according to standard protocols, then cells were plated onto collagen-coated glass coverslips and allowed to extend neurites for 24 hours in media (Neurobasal medium, 50mM pencillin-streptomycin (PS), 0.2% FBS, 0.5mM glutamine, 1 × B-27 supplement, 0.2% glucose, 10ng/ml glial cell line–derived neurotrophic factor). PTX, EQ and its derivatives, or vehicle controls were added to the wells for another 24-hour incubation. DRG cells were fixed with 4% paraformaldehyde and stained with anti–βIII-tubulin antibody to delineate the axons. Axon lengths were measured in multiple fields using a random sampling method as described.[9]

To evaluate the impact of EQ on PTX's ability to kill breast cancer cells, conditions for culturing 4 cancer cell lines and measuring the ATP levels were optimized for the 96-well plate format. Briefly, 1,500 cells/well in media (MDA-MB-231 in Dulbecco modified Eagle medium [DMEM] with 10% FBS; MCF-7 in DMEM with 10% FBS; TATD in Roswell Park Memorial Institute medium with 10% FBS; SUM159 in DMEM/F-12 [250ml/250ml] with 5% FBS, 500μl of 10mg/ml insulin and 25μl of 10mg/ml hydrocortisone) were plated in 96-well plates for 24 hours. Constant concentrations of PTX with or without EQ were added to the wells for another 24 hours. Cellular ATP levels were measured using ViaLight Plus kit (Cambrex) according to manufacturer's protocol on LMax (Molecular Devices).

All validation experiments and experiments involving primary DRG neurons or cancer cells were performed in triplicate and repeated at least twice. Statistical analysis was done using analysis of variance. Correction for multiple comparisons was completed with Fisher protected least significant difference.

In Vivo Neuroprotection and Tumor Burden Studies

Peripheral neuropathy was induced in adult male AJ strain of mice by tail vein intravenous injections of PTX (25mg/kg) on days 1, 3, and 5 as described previously.[10] Vehicle control (saline) or different doses of EQ were administered on a daily basis by intraperitoneal administration starting on day 1 with the first dose of PTX (n = 10 animals per group). Two days before the start of the study, baseline thermal sensation and tail sensory nerve electrophysiology were carried out. Average paw withdrawal latency was recorded using an IITC Life Science (Woodland Hills, CA) Analgesia Meter according to the Hargreaves method. Orthodromic tail sensory nerve conduction studies were done according to standard methods. Recording electrode was placed at the base of the tail, and the stimulating electrode was placed 5cm distally. Sensory nerve action potential amplitude was recorded as the average of 20 stimulations, and conduction velocity was calculated. Two weeks after the last dose of PTX (ie, day 19) of the study, repeat thermal sensation and tail sensory nerve electrophysiology were completed and medial plantar footpads were harvested for determination of intraepidermal nerve fiber densities. Two-millimeter punch biopsies of the footpads were fixed in paraformaldehyde-lysine-periodate fixative, and then stained with a pan-axonal marker anti-PGP antibody (Biogenesis, Kingston, NH; catalog No. 7863-0504). The number of intraepidermal nerve fibers was determined in 6 to 10 sections for each animal and average density was calculated as previously described.[11] The study was repeated once more as an independent validation of the findings from the first study.

To examine the effect of EQ on PTX's ability to reduce tumor burden in vivo, we used a mouse model in which breast cancer cell line SUM-159 (3×10^6 tumor cells suspended in phosphate-buffered saline in a final volume of 0.15ml) was injected subcutaneously into adult male nude mice.[12] When the tumor size reached 5mm in diameter, the animals were randomly assigned to PTX or PTX with EQ groups. PTX was administered at a dose of 20mg/kg intravenously on days 1, 4, and 8, and EQ was given by intraperitoneal administration on a daily basis for 3 weeks. At the end of 3 weeks, animals were euthanized and tumor size and weight were measured (n = 5 per group).

RNA Inhibition Using siRNA

Transfection of 50B11 DRG neuronal cells with various siRNA plasmids was carried out according to the manufacturer's protocol. 50B11 DRG neuronal cells were plated in 6-well plates in culture medium (Neurobasal medium, 10% FBS, 0.5mM glutamine, 1 × B-27 supplement, 0.2% glucose) without antibiotics overnight and allowed to reach 50 to 70% confluence. On the second day, 1pmol siRNA (Ambion, Austin, TX) oligomer was diluted in 250μl Opti-MEM media, and 5μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in another 250μl of Opti-MEM media. After 5 minutes of incubation, dilutions were combined and incubated for 20 minutes at room temperature, and the oligomer–Lipofectamine 2000 complexes were added

to each well. Then 1.5ml Opti-MEM media was added and gently mixed. In 4 hours after the transfection, culture media was replaced by culture media containing blasticidin (5μg/ml), and cells were grown for 72 hours.

Protein Isolation, Protein Electrophoresis (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis), and Western Blotting

These assays were performed according to standard protocols and instructions of the manufacturers of specific kits. Total protein was extracted from 50B11 cells using M-PER Mammalian Protein Extraction Reagent (Invitrogen) in the presence of protease inhibitor (Thermo Scientific, Waltham, MA). The measurements of total protein concentrations were performed using BCA kits (Thermo Scientific) on SPECTRAmax 340PC (Molecular Devices). Samples (30μg of total protein per well) and standards (SeeBlue Plus2 Pre-Stained Standard; Invitrogen) were loaded onto the wells (Ready Gel 4–15% Tris-HCl, 50μl-1gel; Bio-Rad, Hercules, CA). The gel was run at 100V for 1.5 hours, and then transferred to polyvinylidene difluoride membrane (Bio-Rad) according to the manufacturer's protocol. The membrane was blocked at room temperature for 1 hour in a blocking buffer (5% milk), then incubated with primary antibody (Abcam [Cambridge, MA] or StressMarq Biosciences [Victoria, BC, Canada]) at 1:1,000 dilution at 4°C overnight (please see Supplementary Material for full details of all antibodies and catalogue numbers). This was followed by 3 washes and incubation with the second antibody at 1:1,000 dilution at room temperature for 1 hour. After 3 washes and development in ECLTM Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK), the membrane was covered in transparent wrap and exposed to x-ray film according to the manufacturer's recommendation.

Colorimetric Determination of ATPase Activity of Hsp90

The assay procedure was based on a published paper.[13] On the day of use, the malachite green reagent was prepared. It contained malachite green (Sigma, St Louis, MO; 0.0812%, wt/vol), polyvinyl alcohol (Sigma; 2.32%, wt/vol), ammonium molybdate (Sigma; 5.72%, wt/vol, in 6M HCl), and UltraPure Distilled Water (Invitrogen), mixed in the ratio 2:1:1:2. The reagent was initially dark brown, and then changed to a golden yellow on standing for 2 hours at room temperature, becoming ready for use. The assay buffer (pH 7.4) was made with 100mM Tris-HCl, 20mM KCl, and 6mM $MgCl_2$. Test compounds were dissolved in 100% (vol/vol) DMSO to give a stock concentration of 10mM. This solution was diluted to 6 appropriate concentrations with test compounds in the assay buffer. Five microliters of each compound solution was added to each well of the Perkin Elmer (Waltham, MA) 96-well assay plate. The first 2 rows of the 96-well plate contained DMSO only, representing the control and background wells. ATP (Sigma) was dissolved in the assay buffer to make a stock solution with a concentration of 2.5mM, which was placed on ice. A 10μl ATP solution was added to each well to give a final assay concentration of 1mM. Just before use, recombinant Hsp90 protein (StressMarq Biosciences) was placed on ice and suspended in cold assay buffer to make a stock solution with a concentration of 0.50mg/ml. Ten microliters of the stock Hsp90 solution was added to each well (except for the background wells that received 10μl of assay buffer), giving a final assay volume of 25μl. The plates were shaken (approximately 5 minutes) using a plate shaker (VWR [Radnor, PA] DV-150 Waver) sealed with plastic film and incubated at 37°C for 3 hours. To stop the incubation, 50μl of the malachite green reagent was added to each well and the plate was shaken again. After addition of 10μl of 34% sodium citrate to each well, the plate was shaken again and left to stand at room temperature for 20 minutes. The absorbance at 620nM was measured using SpectraMAX 340PC (Molecular Devices). All experiments were performed in duplicates and repeated at least twice.

Fluorescence-Quenching Assay

Fluorescence intensity was recorded on a SpectraMax Gemini XS plate reader (Molecular Devices) using a 96-well black polymerase chain reaction plate (Thermo Scientific). Compounds (10mM in ethanol) were diluted with 50mM Tris-HCl (pH 7.4) to obtain a solution with a 15μM final concentration. Aliquots of proteins were added to the compounds solution and mixed with a micropipette by sucking the solution gently up and down, then florescence intensities (λ_{ex} = 350nM, λ_{em} = 450nM) were measured at 25°C according to published methods.[14,15] Equilibrium dissociation constant K_d was obtained by fitting the data using the nonlinear least squares option of Prism software (GraphPad Software, La Jolla, CA). All experiments were completed in duplicates and repeated at least 3×.

Binding Studies Using Affinity Capture Chromatography

An AminoLink Plus Immobilization Kit was purchased from Thermo Scientific. The assay procedure was based on a published paper[16] and was carried out according to the manufacturer's protocol. AminoLink Plus Coupling resin was incubated overnight in a solution of 200μmol EQ-der.6 (6-(5-amino)-pentoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline, dissolved in DMSO: pH 7.2, coupling buffer 20:80). Then the resin was equilibrated to pH 7.2 in phosphate buffer containing 150mM NaCl. To characterize the compound linked to AminoLink, we used high-performance liquid chromatography to measure the coupling efficiency. Residue and uncoupled compound in solution were determined; the amount of coupled fraction was calculated. Resins were treated with a 50mM sodium cyanoborohydride solution (40μl in 2ml quenching buffer) to block the residual active sites on the resin surface. After several washing steps, total protein extracts from 50B-11DRG neuronal cell line and primary mouse DRGs (obtained from adult mice weighing about 20g by unilateral excision of the L3, L4, L5, and L6 DRGs) were passed through these affinity columns and extensively washed to remove nonspecifically bound proteins. Elution was performed with 1mM EQ-der.6 in elution buffer. The elute was resolved by sodium dodecyl sulfate–

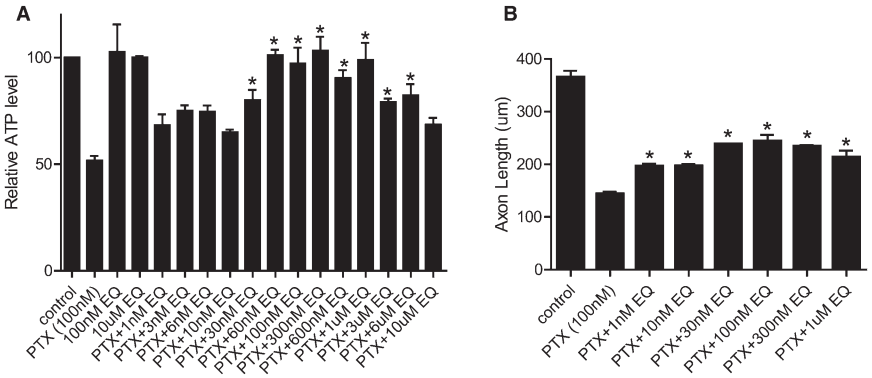

FIGURE 1: Neuroprotection by ethoxyquin (EQ) in vitro. (A) 50B11 dorsal root ganglion (DRG) neuronal cells were differentiated, then exposed to paclitaxel (PTX) with or without various concentrations of EQ. Adenosine triphosphate (ATP) levels were measured after 24 hours. (B) Primary rat DRG neurons were grown in culture and allowed to extend their axons for 24 hours. Then they were exposed to PTX or EQ for another 24 hours. Cells were fixed and stained with βIII-tubulin, and axon lengths were measured. EQ partially prevented distal axonal degeneration induced by PTX (n = 8–10 per group; *$p < 0.05$ compared to PTX alone).

polyacrylamide gel electrophoresis (SDS-PAGE), and then revealed by Pierce Silver Stain (Thermo Scientific) and Coomassie brilliant blue. Bands of interest were cut out of the gel and analyzed by nano-LC ESI/MS/MS Orbitrap Velos fast time frequency transform (FTFT; Thermo Scientific). Proteins were searched and identified in protein database, then analyzed on Scaffold 3.

Identification of Hsp90 Client Proteins Modulated by EQ

Recombinant Hsp90 tagged with His (Hsp90ab1-His) was purchased from Abcam, and linked to His Pur TM Ni-NTA resin (Thermo Scientific) according to the manufacturer's specifications. Half of the Hsp90ab1 coupling resin was incubated at 4°C for 3 hour in a solution of 0.5mg/ml EQ (in 7% ethanol in M-PER with protein inhibitor). After several washings, total protein extracts from 50B11 DRG neuronal cells were incubated with these affinity columns (with or without EQ binding) at 4°C for 4 hours and extensively washed with 10mM pH 7.5 Tris-HCl to remove nonspecifically bound proteins. After additional washing with 10mM pH 7.5 Tris-HCl containing 500mM NaCl, elution was performed with 400μl 100mM glycine (pH 2.8). Elutes were resolved by SDS-PAGE gel and then revealed by Pierce Silver Stain. The bands of interest were cut from the gel and analyzed by nano-LC ESI/MS/MS Orbitrap Velos FTFT. Proteins were searched and identified in protein databases, and then analyzed on Scaffold 3.

Impact of EQ or EQ-Der.2 on Binding between Hsp90 and 3 Client Proteins

Recombinant Hsp90ab1-His (Abcam) was linked to His Pur TM Ni-NTA resin (Thermo Scientific) according to the manufacturer's specifications, then incubated at 4°C for 2 hours with various concentrations of EQ or inactive analogue, EQ-Der.2 (EQ = 0, 10nM, 30nM, 100nM, 300nM, 1,000nM in ethanol with protein inhibitor). After several washings, recombinant ATXN2, SF3B2, and p54/nrb proteins (Novus Biologicals, Littleton, CO) were incubated with these affinity columns (with or without EQ binding) at 4°C overnight, then extensively washed with M-PER with protein inhibitor to remove nonspecifically bound proteins. The beads were incubated in 100μl loading buffer at 95°C for 5 minutes, then the buffer containing protein was resolved by SDS-PAGE gel and revealed by Western blotting.

Results

We used an immortalized DRG neuronal cell line, 50B11, to screen for compounds that prevented toxicity induced by PTX, zalcitabine (an antiretroviral drug), and capsaicin. The rationale for using 3 different neurotoxins that cause sensory axonal degeneration was to identify compounds that acted downstream of the potential specific mechanism of action of toxicity of each compound. We carried out the screen as previously described[8] using the 2,000-compound library Spectrum collection (www.msdiscovery.com). Out of the screen, we identified 32 compounds that provided 50% or more neuroprotection. Further evaluation using a wider dose range of each compound and validation assays using primary rat DRG neuron–Schwann cell cocultures showed the best proper dose–response curves for EQ, with a peak efficacy range at 30 to 300nM (Fig 1).

To understand the relative contributions of different parts of its structure to neuroprotection, we prepared derivatives of EQ as shown in Supplementary Figure 1. Most of the EQ derivatives retained neuroprotection against PTX-induced neurotoxicity in the DRG sensory neuronal line except derivatives 2 and 3, which did not show any appreciable dose–response relationships (Supplementary Fig 2).

Next we examined EQ's potential for neuroprotection in a mouse model of chemotherapy-induced peripheral neuropathy (CIPN) using intravenous PTX administration. As described previously,[10] a short course of intravenous PTX administration every other day

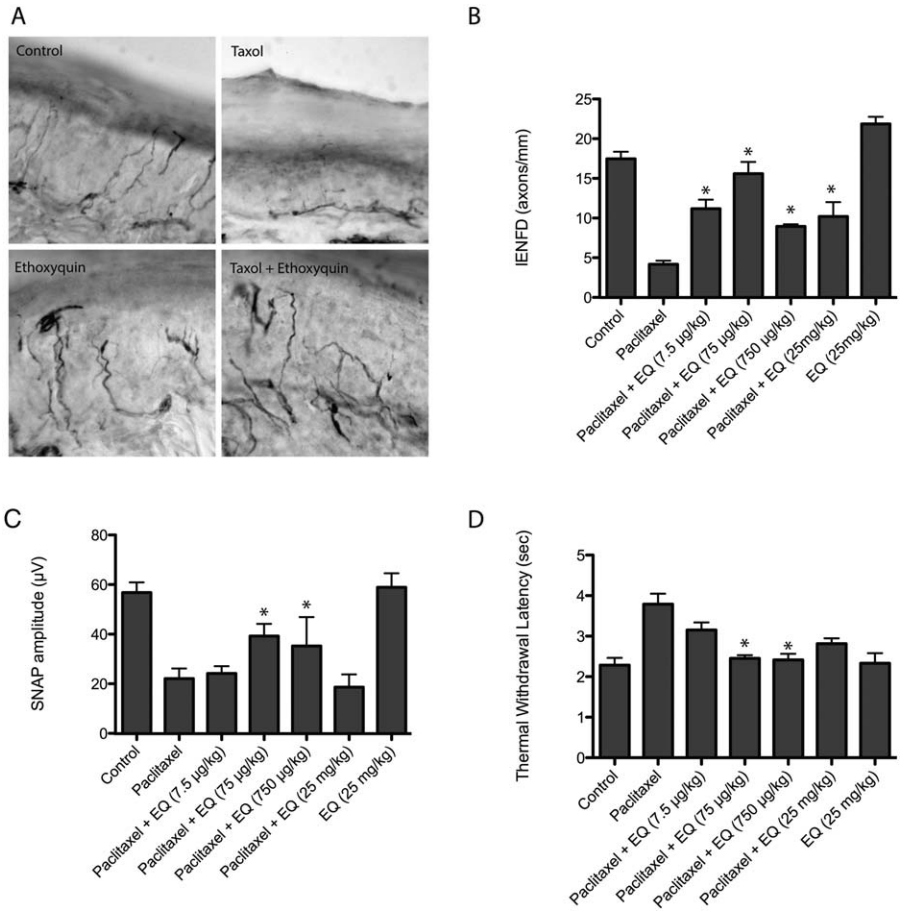

FIGURE 2: Neuroprotection by ethoxyquin (EQ) against paclitaxel-induced peripheral neuropathy. Coadministration of EQ with paclitaxel partially prevented development of peripheral neuropathy as assessed by (A, B) intraepidermal nerve fiber density (IENFD), (C) sensory nerve action potential (SNAP) amplitude, and (D) thermal withdrawal latency (n = 10–20 per group, *p < 0.05 compared to PTX alone).

(25mg/kg on days 1, 3, and 5) causes a mild sensory neuropathy that mimics the early stages of CIPN. Because CIPN is an ideal clinical target for development of neuroprotection (one can administer the potential neuroprotective drug before the axonal injury takes place), we elected to test EQ in a preventative study design; we administered it starting on day 1 and continued for another 14 days after the last PTX dose. The primary endpoint of the study was change in intraepidermal nerve fiber density in the hindlimb plantar footpads. As shown in Figure 2, EQ provided a dose-dependent neuroprotection. Although all 3 doses tested provided partial neuroprotection against reduction in intraepidermal nerve fiber density, the peak efficacy was with the 75μg/kg dose. A similar neuroprotection was observed with the secondary outcome measures, reduction in sensory nerve action potential amplitude and thermal hypoalgesia.

For EQ to be of value in a clinical setting, it had to have no effect on PTX's ability to kill cancer cells. We evaluated this both in vitro and in vivo, as shown in Figure 3. EQ did not block PTX's ability to induce cell death in 4 different breast cancer cell lines (a kind gift of Dr. S. Sukumar, Johns Hopkins School of Medicine), even when administered at doses higher than those that protected against PTX-induced neurotoxicity. These 4 different cell lines were chosen because they have different receptor properties and different susceptibility to PTX. We then took 1 of these tumor cell lines (SUM-159) and injected it into mice to induce tumors. After formation of tumors, we administered PTX according to established protocols[12] and monitored tumor growth. EQ did not affect PTX's ability to reduce tumor growth (see Fig 3E, F).

Because the initial screen was a phenotypic screen rather than a molecular mechanism–based screen, we did not know exactly how EQ provided neuroprotection. To identify the mechanism of its action, we synthesized a novel EQ analogue (derivative 6) to attach and covalently bound it to a resin column. Then we passed lysates from rat DRG neuronal cell line, rat sciatic nerve, or rat DRGs through the column, eluted the bound proteins, and analyzed with mass spectrometry (Supplementary Fig

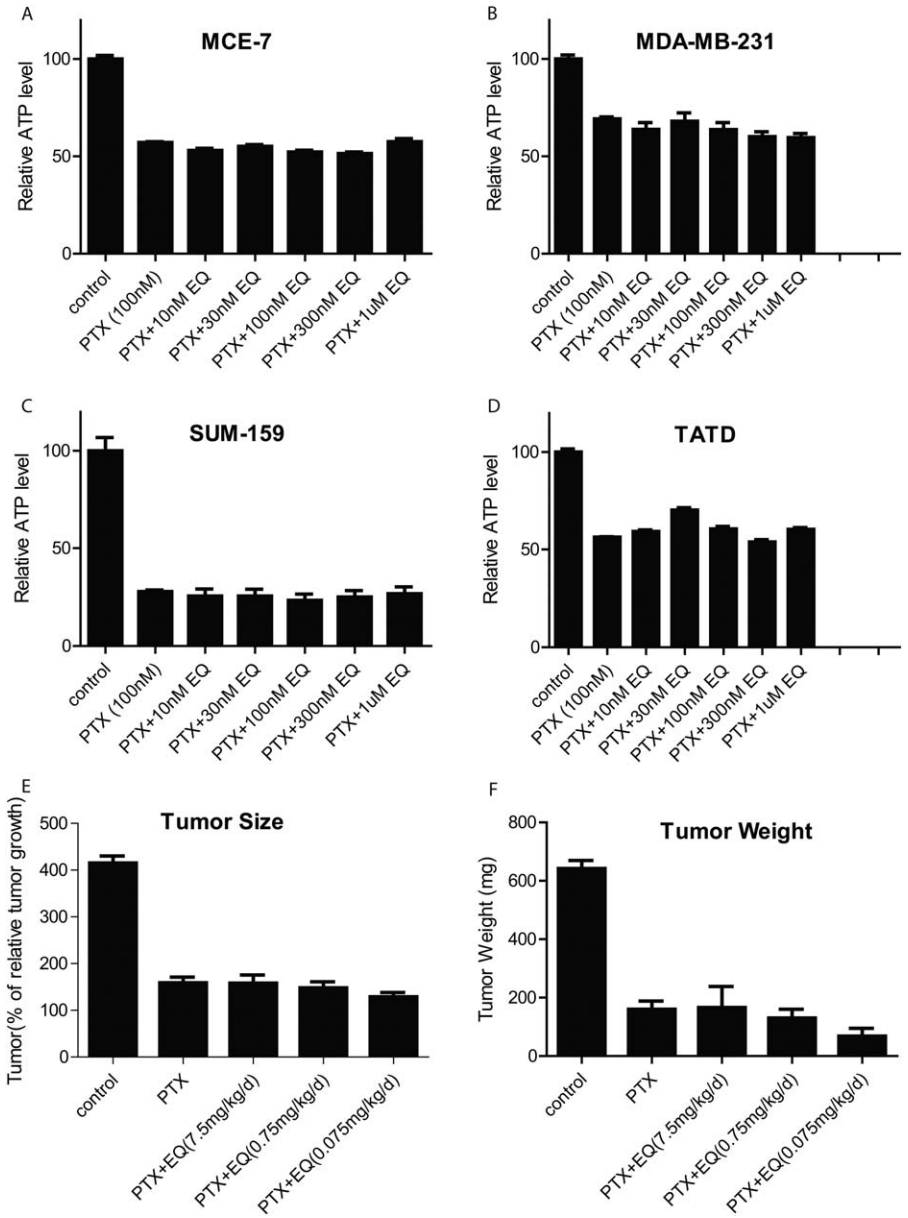

FIGURE 3: Ethoxyquin (EQ) does not block the chemotherapeutic effects of paclitaxel (PTX). When 4 different breast cancer cell lines (A–D) were grown in culture, PTX reduced cell viability by 35 to 75%; addition of EQ at various doses did not have any appreciable effect (n = 8–10 per group). Similarly, in an animal model, EQ did not prevent PTX's ability to reduce tumor size (E) or tumor weight (F; n= 5 per group). ATP = adenosine triphosphate.

3). There were 8 proteins that bound to the column. To identify which of these proteins played a key role in mediating the efficacy of EQ, we used an RNA inhibition strategy to downregulate levels of each protein that bound to EQ and validated the reduction in protein levels by Western blotting (see Supplementary Fig 3B and C). Then we asked whether EQ-mediated neuroprotection was lost when expression of a given protein level was downregulated. These studies identified that only when Hsp90 level was downregulated was neuroprotection provided by EQ no longer seen (Fig 4). When other protein levels were downregulated, there was no diminishment of neuroprotection by EQ (Supplementary Fig 4).

Identification of Hsp90 as a potential mediator of neuroprotection by EQ was an interesting finding, given that there is already an effort in the field to identify Hsp90 inhibitors as anticancer drugs and potentially as neuroprotective compounds.[13,17–22] Hsp90 exits as a homodimer and contains 3 domains, including a 25kDa N-terminal ATP-binding domain, a 12kDa C-terminal dimerization domain, and a 35kDa middle domain.[18] Current Hsp90 inhibitors act primarily by binding to the N-terminal of Hsp90 and inhibiting its ATPase activity.[13,22] We first

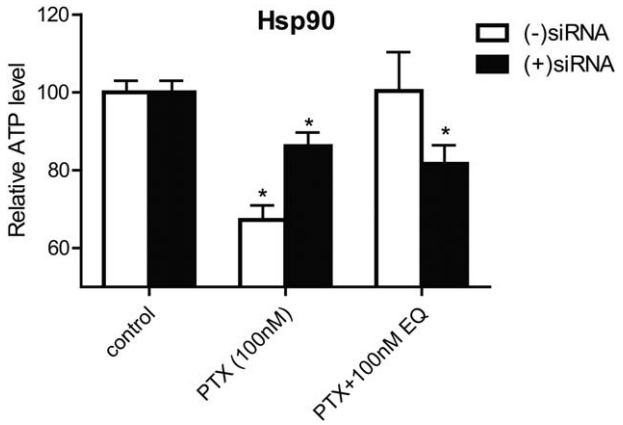

FIGURE 4: Loss of neuroprotection by RNA interference of Hsp90. When Hsp90 protein levels were reduced by siRNA, neuroprotection provided by ethoxyquin (EQ) against toxicity of paclitaxel (PTX) was lost. A negative control siRNA did not have any appreciable effects (n = 8–10 per group; *$p < 0.05$ compared to control cultures). ATP = adenosine triphosphate.

examined the binding efficiency of EQ to recombinant Hsp90 using the fluorescence quenching method. As shown in Figure 5A, EQ bound to Hsp90 with a K_d of 280nM, well within the effective dose range in our in vitro neuroprotection assays. Then we tested whether EQ inhibited ATPase activity of Hsp90. As shown in Figure 5B, EQ did not have any effect on ATPase activity of Hsp90 across a very wide dose range. A review of the literature suggested that PTX can potentially bind to Hsp90,[23] but the relevance of this observation in mediating the axonal toxicity of PTX is unclear. Nevertheless, as EQ could potentially displace PTX from Hsp90 and prevent its axonal toxicity, we investigated this possibility using a fluorescence-quenching assay. As shown in Figure 5C, when EQ was present at 1.5μM concentration, there was no displacement of EQ across a wide dose range of PTX, suggesting that EQ and PTX likely bind to different sites on Hsp90.

Because these findings indicated that EQ was acting through Hsp90 by modulating another activity, we evaluated the possibility that EQ might be altering its chaperone function. To find out which proteins that normally bind to Hsp90 are displaced by EQ, we prepared a column in which His-tagged Hsp90 was bound and treated with either EQ or buffer before a lysate of DRG neuronal cell line was passed through. Then we resolved the eluted fraction by gel electrophoresis, excised the protein bands, and analyzed by mass spectrometry. Among the many proteins that were eluted, only the following 3 proteins were displaced from binding to Hsp90 by pretreatment with EQ: ATXN2 (ataxin-2), p54/nrb (an RNA recognition domain-containing protein), and SF3B2 (splicing factor 3b, subunit 2; Supplementary Fig 5).

To confirm changes in levels of these proteins in the presence of EQ, we treated DRG neuronal cell lines for 24 hours with and without PTX and/or EQ and measured protein levels by Western blotting (Fig 6). As shown in Figure 6A, there was no effect of PTX alone

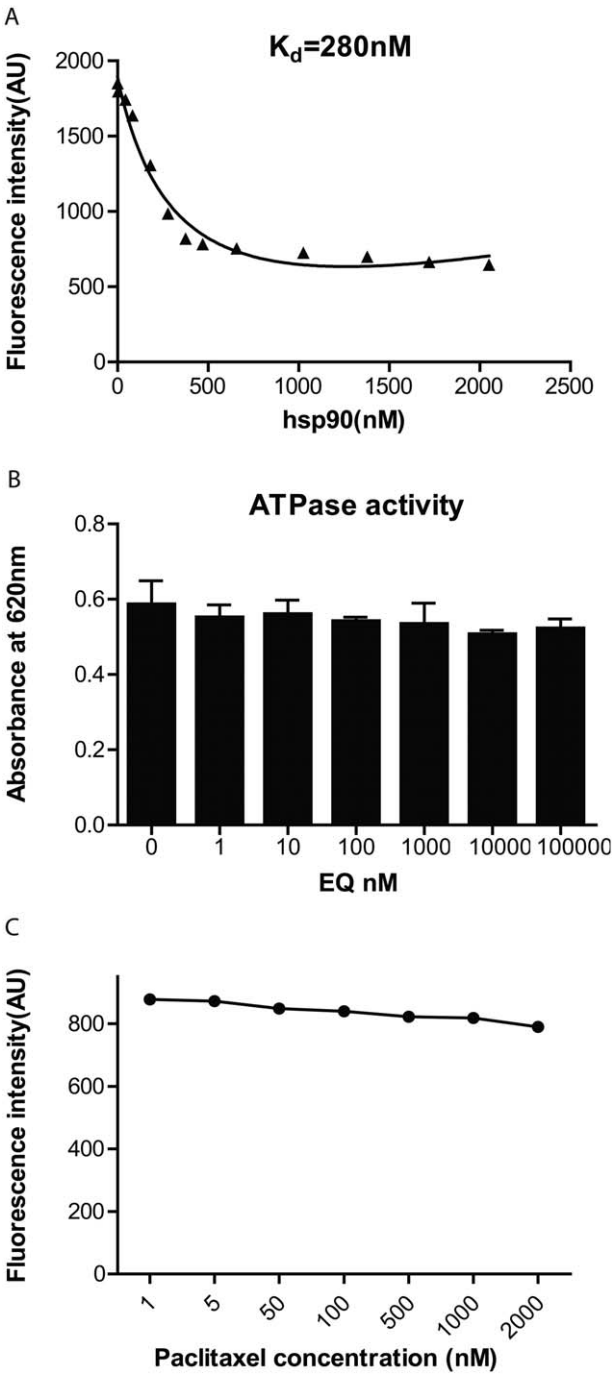

FIGURE 5: Binding of ethoxyquin to Hsp90. (A) Ethoxyquin (EQ) binds to recombinant Hsp90 with an equilibrium dissociation constant (K_d) of 280nM using the fluorescence quenching method. (B) EQ does not alter the adenosine triphosphatase (ATPase) activity of Hsp90 across a wide dose range. (C) EQ does not alter the binding of paclitaxel to recombinant Hsp90. AU, arbitrary units.

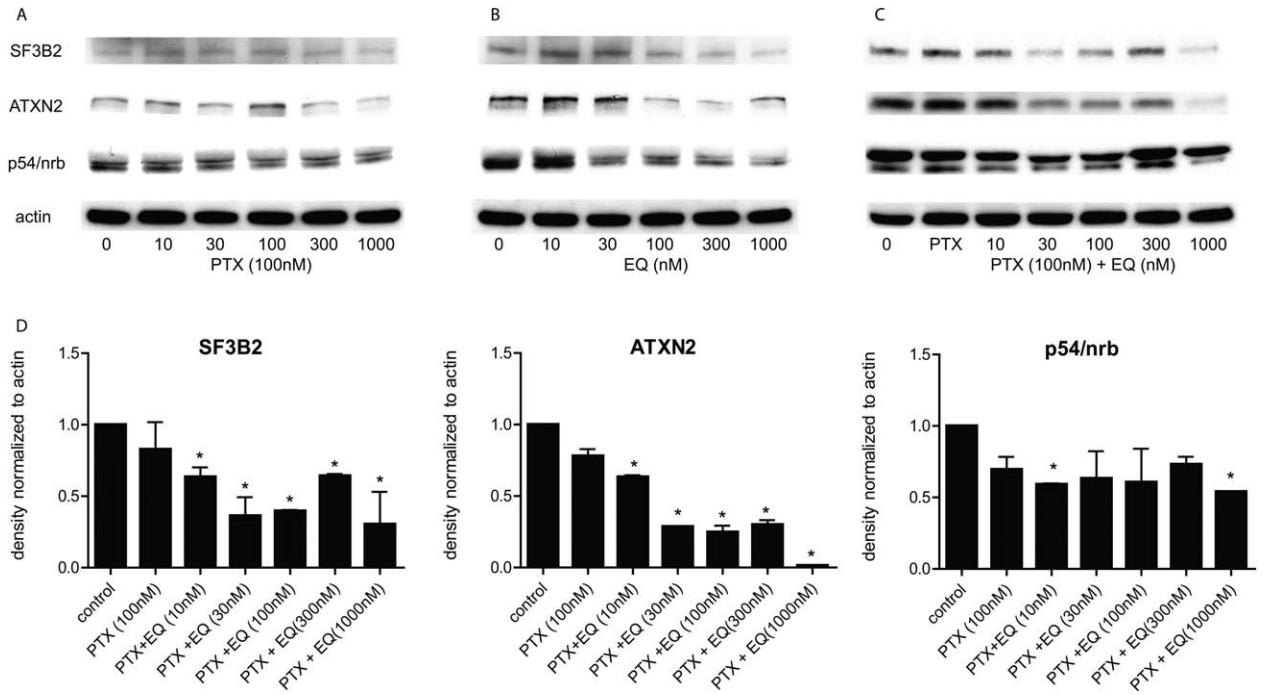

FIGURE 6: The effect of ethoxyquin on 3 candidate client proteins of Hsp90. Representative Western blots are shown of each candidate protein in 50B11 dorsal root ganglion neuronal cultures when treated with paclitaxel (PTX) alone (A), ethoxyquin (EQ) alone (B), or both in combination over 24 hours (C). (D) Densitometric ratios of proteins levels in cultures treated with PTX and EQ (bar graphs denote means of density measurements from 3 separate Western blots from 3 separate experiments per condition).

on the levels of all 3 proteins, but incubation of DRG neuronal cells with EQ (with or without PTX) resulted in a dose-dependent reduction in levels of all 3 proteins. We then confirmed this interaction between EQ, Hsp90, and 3 of its client proteins in a binding assay using resin-bound His-Hsp90 and recombinant ATXN2, p54/nrb, and SF3B2 (Fig 7). When this column was preincubated with EQ, binding of all 3 recombinant proteins was inhibited in a dose-dependent manner.

Although these observations led us to think that neuroprotection by EQ was mediated through Hsp90 by lowering the cellular levels of these 3 proteins, the inactive analogues of EQ allowed us to ask which of these 3 client proteins of Hsp90 was truly relevant for neuroprotection by EQ. We repeated the same experiment as outlined above (see Fig 7) with derivative 2 of EQ in place of EQ and found that levels of ATXN2 and SF3B2 were not altered in the presence of EQ-der.2, suggesting that 1 or both of these proteins is likely to play a key role in mediating the neuroprotective effects of EQ (Fig 8). Combined, these findings indicated that EQ might act as a modulator of chaperone activity of Hsp90 and provide neuroprotection.

Discussion

In this study, we identified EQ as a potential neuroprotective therapy against PTX-induced peripheral neuropathy and showed that its mechanism of action is likely to be mediated by inhibiting the chaperone activity of Hsp90. Furthermore, we identified ATXN2 and SF3B2 as potential client proteins of Hsp90 whose levels are modulated by binding of EQ to Hsp90.

EQ (1,2-dihydro-6-ethoxy-2,2,4,trimethylqyuinoline) is a synthetic antioxidant first approved by the US Food and Drug Administration for use in animal food almost 50 years ago. Later mice fed a diet supplemented with EQ were found to live longer than littermates.[24] Other studies suggested that EQ and similar antioxidants may inhibit chemical carcinogenesis (reviewed in Comfort et al,[24] Wattenberg[25]). However, these observations were never carried over to human studies, and the primary use of EQ is still as a supplement in animal food.

Although earlier studies focused on antioxidant properties of EQ, because we started with an unbiased phenotypic screen we initially had no idea about EQ's mechanism of action. The peak dose range that we found effective (100–300nM) provided a clue that this was not likely to be related to antioxidant properties of EQ, which are in the micro- to millimolar range.[26,27] We again used an unbiased approach to identify proteins that interacted with EQ and found that EQ binds to Hsp90 with high affinity.

Heat shock proteins are a class of functionally related proteins involved in folding and unfolding of other proteins and act as "quality control checkpoints"

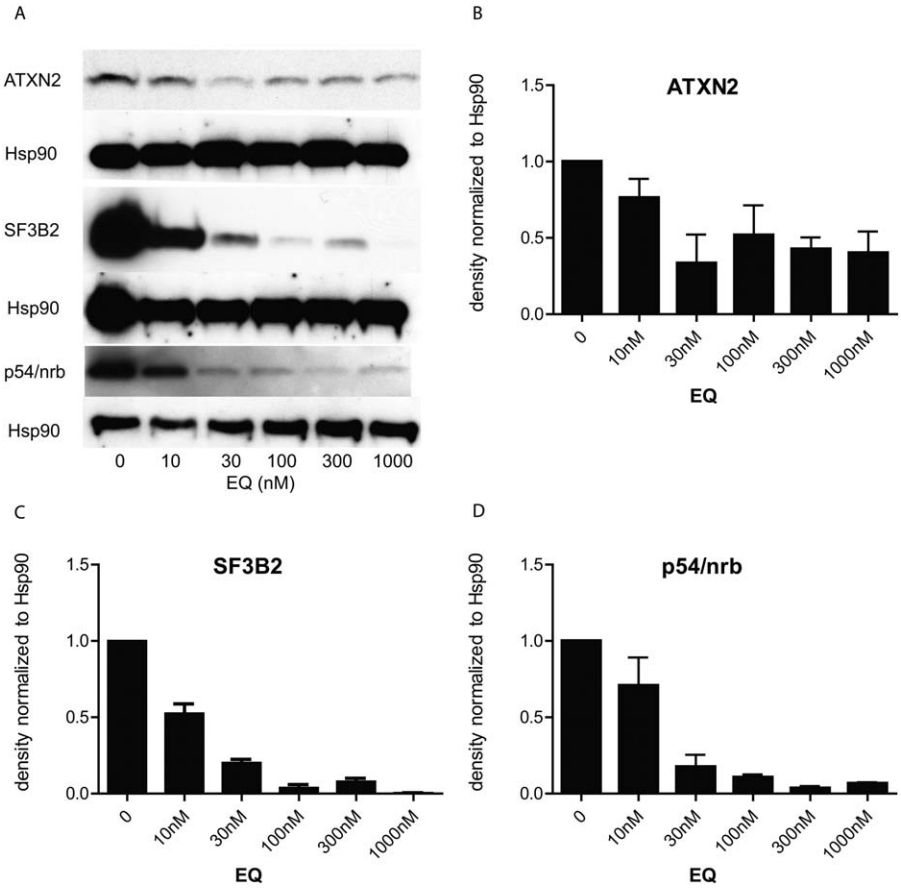

FIGURE 7: The effect of ethoxyquin (EQ) on binding of 3 candidate client proteins to recombinant Hsp90. (A) Representative Western blots of 3 candidate client proteins bound to recombinant the Hsp90-his column when incubated with various concentrations of EQ. Bar graphs denote averages of 3 experiments for ATXN2 (B), SF3B2 (C), and p54/nrb (D).

for protein integrity in the cell.[28,29] They do this by acting as chaperones for their client proteins; they help stabilize partially unfolded proteins, transport them across membranes within a cell, help establish proper protein conformation, prevent unwanted protein aggregation, and carry old proteins destined for degradation to a proteasome in the cell. They are highly expressed in every cell, comprising up to approximately 1 to 2% of the total protein in unstressed cells. This percentage increases to 4 to 6% of the total protein in cells that are stressed, such as during elevated temperatures, inflammation, or infection.

Among heat shock proteins, Hsp90 has been implicated in pathogenesis of various cancers and is an active drug target because some of its client proteins play critical roles in oncogenesis and metastasis (reviewed in Hong et al[30]). In addition, Hsp90 has been implicated in the pathogenesis of various neurodegenerative diseases and will likely be a target for disease-modifying therapies.[21,31] It was coincidental that we identified Hsp90 as the binding partner for EQ through an unbiased biochemical approach. The exact role of Hsp90 and its mechanism of action in cancer and neurodegenerative diseases are likely to be complicated and may involve multiple disparate modes of activities.

To tease out the relationship between EQ and Hsp90, we again used an unbiased biochemical assay to find which proteins' binding to Hsp90 was altered when EQ was allowed to bind to Hsp90 first. This resulted in identification of 3 proteins, ATXN2, p54/nrb, and SF3B2; all have been implicated in RNA metabolism. Furthermore, the inactive analogue of EQ allowed us to narrow down the potential candidate client proteins to ATXN2 and SF3B2.

Of these 2 proteins, only ATXN2 is known to play a role in neuronal function. Expansion of the trinucleotide CAG repeat in ataxin-2 is a frequent cause of autosomal dominant spinocerebellar ataxia.[32] Furthermore, ataxin-2 is abnormally localized in spinal cord motor neurons of amyotrophic lateral sclerosis (ALS) patients, and intermediate-length polyglutamine expansions in the ataxin-2 gene are associated with increased risk of ALS.[33] How ataxin-2 mediates neurotoxicity and how reductions in ataxin-2 levels are involved in neuroprotection are

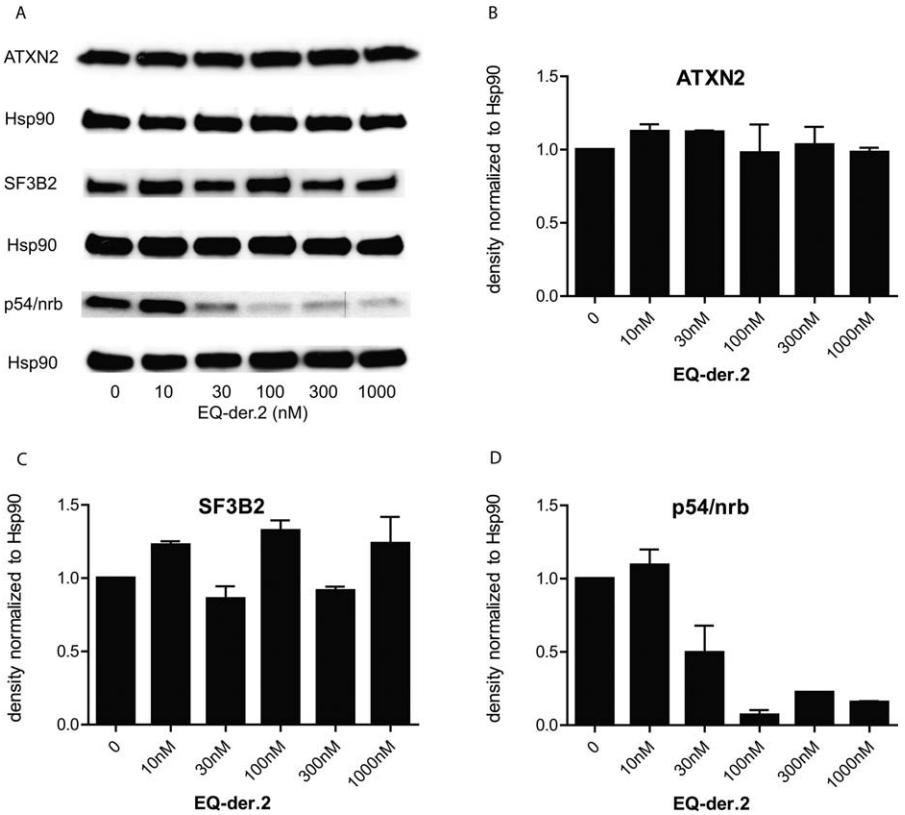

FIGURE 8: The effect of EQ-der.2 (inactive analogue of ethoxyquin [EQ]) on binding of 3 candidate client proteins to recombinant Hsp90. (A) Representative Western blots of 3 candidate client proteins bound to the recombinant Hsp90-his column when incubated with various concentrations of EQ-der.2. Bar graphs denote averages of 3 experiments for ATXN2 (B), SF3B2 (C), and p54/nrb (D).

unclear. It is possible that this effect is mediated by altering the RNA processing of other genes. The other protein, SF3B2, that is affected by EQ is also involved in RNA processing as a component of splicing complex SF3B.[34] Which of these 2 Hsp90 client proteins is responsible for the actual neuroprotection by EQ remains unclear, but they could be acting in concert in modulating RNA processing.

Alterations in RNA processing may be a common pathway underlying many neurodegenerative diseases.[35] Recent studies identified mutations in TDP-43 (TAR DNA-binding protein 43) and FUS (fused in sarcoma) that result in altered RNA processing and cause frontotemporal lobar degeneration or motor neuron diseases.[36–38] A more recent finding of hexanucleotide repeat expansion in C9ORF72 as a major cause of sporadic motor neuron disease reinforced the key role altered RNA processing may play in pathogenesis of neurodegenerative diseases. Thus, modulation of RNA processing may offer a common pathway for neuroprotection and perhaps even prevention of disease progression in many neurodegenerative diseases.

Modulation of Hsp90 client protein activity may be a common pathway for neuroprotection irrespective of its ATPase activity. When we tested a traditional Hsp90 inhibitor, 17-NAAG, that is in clinical trials for cancer therapy[22] or 17-DMAG, we did not find any neuroprotection against PTX-induced neurotoxicity in the DRG neuronal cell line (Supplementary Fig 6). Another inhibitor of C-terminal activity of Hsp90 was recently identified as potential therapy for diabetic neuropathy.[39] This drug, KU-32, was shown to alter levels of other client proteins of Hsp90, especially p53. It is unclear whether KU-32 would prove to be neuroprotective in PTX-induced peripheral neuropathy models, but in preliminary studies we have seen that EQ does provide neuroprotection against diabetic neuropathy in a model of type-1 diabetes induced with streptozotocin (data not shown).

Although we believe EQ and analogues or other chemicals that act on Hsp90 chaperone activity may be effective in preventing the distal axonal degeneration seen in PTX-induced peripheral neuropathy, we do need to acknowledge some shortcomings of our study. First, we used a relatively mild in vivo model of CIPN that was short in duration and did not induce any motor deficits. The neuroprotective effects we observed with EQ may not be seen in more severe or chronic models of PTX-

induced peripheral neuropathy.[40] Furthermore, how our observations with Hsp90 chaperone activity link to previously published work demonstrating neuroprotective effects of erythropoietin,[10] calpain inhibition,[41] *Wlds* (Wallerian degeneration slow) mutation,[42] alpha-lipoic acid,[43] acetyl-L-carnitine,[44] olesoxime,[45] or glutamate carboxypeptidase inhibition[46] remains to be investigated. In our study, reducing levels of Hsp90 protein itself had a partial neuroprotective effect (see Fig 4), making it difficult to appreciate the full effect of reduction of Hsp90 levels on neuroprotection mediated by EQ. It is possible that reduced levels of Hsp90 led to diminished processing of its client proteins and reduced their levels similarly to what is achieved by binding of EQ to Hsp90.

In summary, inhibition of C-terminal activity or modulation of chaperone activity of Hsp90 may be a common pathway for neuroprotection for disorders characterized by distal axonal degeneration. EQ and its novel analogues may prove to be a new class of drugs for neuroprotective therapy for peripheral neuropathies.

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Potential Conflicts of Interest

J.Z., W.C., A.H.: patents, Compounds for Treating Peripheral Neuropathies and Other Neurodegenerative Disorders. A.H.: consultancy, Teva Pharmaceuticals, BMS, Unilab; grants/grants pending, Acorda Therapeutics.

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