Ethoxyquin Prevents Chemotherapy-Induced Neurotoxicity via Hsp90 Modulation

Jing Zhu Ph.D., Weiran Chen M.D., Ruifa Mi M.D., Ph.D., Chunhua Zhou B.S., Nicole Reed M.S. and Ahmet Höke M.D., Ph.D.

Departments of Neurology and Neuroscience, Johns Hopkins School of Medicine

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Correspondence:
Ahmet Höke MD, PhD
Johns Hopkins School of Medicine
855 N. Wolfe Street, Rangos 248
Baltimore, MD 21286
Phone: 410-955-2227
Fax: 410-502-5459

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Abstract

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 pre-administered 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 demonstrate that ethoxyquin acts by modulating the chaperone activity of heat shock protein 90 (Hsp90) and blocking the binding of two 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.
Introduction

Paclitaxel is a chemotherapeutic drug used extensively to treat a variety of solid tumors, including ovarian, breast, and lung cancer. However, like many other chemotherapeutic drugs, paclitaxel can cause severe peripheral neuropathy that affects patients in early stages of treatment and results in long-lasting morbidity. Chemotherapy induced peripheral neuropathy greatly impacts patients’ quality of life and limits the use of higher doses of the drug. Currently, there is no effective therapy to prevent peripheral neuropathy caused by paclitaxel. 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 paclitaxel induced peripheral neuropathy without blocking paclitaxel’s ability to kill tumor cells. Furthermore, we identify heat shock protein 90 (hsp90) as the key molecule mediating the neuroprotective effects of ethoxyquin.

Results

We used an immortalized dorsal root sensory ganglion (DRG) neuronal cell line, 50B11, to screen for compounds that prevented toxicity induced by paclitaxel, zalcitabine (an antiretroviral drug) and capsaicin. The rationale for using three 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 using the 2000-compound library Spectrum collection (www.msdisccovery.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 rat DRG neuron-Schwann cell co-cultures showed the best proper dose response curves for ethoxyquin with a peak efficacy range at 30-300 nM (Figure 1A and 1B).
In order to understand the relative contributions of different parts of its structure to neuroprotection we prepared derivatives of ethoxyquin as shown in Supplementary Figure 1. Most of the ethoxyquin derivatives retained neuroprotection against paclitaxel-induced neurotoxicity in the DRG sensory neuronal line except derivatives 2 and 3, which did not show any appreciable dose-response relationships (Supplemental Figure 2).

Next we examined ethoxyquin’s potential for neuroprotection in a mouse model of chemotherapy induced peripheral neuropathy (CIPN) using intravenous paclitaxel administration. As described previously, a short course of intravenous paclitaxel administration every other day (25 mg/kg on days 1, 3 and 5) causes a mild sensory neuropathy that mimics the early stages of CIPN. Since 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 ethoxyquin in a preventative study design and administered it starting on day 1 and continued for another 14 days after the last paclitaxel dose). The primary end-point of the study was change in intraepidermal nerve fiber density in the hind limb plantar footpads. As shown in Figure 2, ethoxyquin provided a dose-dependent neuroprotection. Although all three doses tested provided partial neuroprotection against reduction in intraepidermal nerve fiber density, the peak efficacy was with 75 µg/kg dose. A similar neuroprotection was observed with secondary outcome measures, reduction in sensory nerve action potential amplitude and thermal hypoalgesia.

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

Since the initial screen was a phenotypic screen rather than a molecular mechanism based screen, we did not know exactly how ethoxyquin provided neuroprotection. In order to identify mechanism of its action, we synthesized a novel ethoxyquin analogue (Derivative 6) in order to attach it to a column 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 (Supplemental Figure 3). There were eight proteins that bound to the column. In order to identify which one of these proteins played a key role in mediating the efficacy of ethoxyquin, we used a RNA inhibition strategy to downregulate levels of each protein that bound to ethoxyquin and validated the reduction in protein levels by Western blotting (Supplemental Figure 3b and 3c). Then we asked if ethoxyquin mediated neuroprotection was lost when expression of a given protein levels was downregulated. These studies identified that only when Hsp90 level was downregulated, neuroprotection provided by ethoxyquin was no longer seen (Figure 4). When other protein levels were downregulated there was no diminishment of neuroprotection by ethoxyquin (Supplemental Figure 4).

Identification of Hsp90 as a potential mediator of neuroprotection by ethoxyquin was an interesting finding given that there is already an effort in the field to identify Hsp90 inhibitors as anti-cancer drugs and potentially as neuroprotective compounds. Hsp90 exits as a homodimer and contains three domains including a 25 kDa N-terminal ATP-binding domain, a 12 kDa C-terminal dimerization domain and a 35 kDa middle domain. Current Hsp90 inhibitors act primarily by binding to the N-terminal of Hsp90 and inhibiting its ATPase activity. We first examined the binding efficiency of
ethoxyquin to recombinant Hsp90 using fluorescence quenching method. As shown in Figure 5a, ethoxyquin bound to Hsp90 with a Kd of 280nM, well within the effective dose range in our in vitro neuroprotection assays. Then we tested whether ethoxyquin inhibited ATPase activity of hsp90. As shown in Figure 5b, ethoxyquin did not have any effect on ATPase activity of Hsp90 across a very wide dose range. A review of the literature suggested that paclitaxel can potentially bind to hsp90 but relevance of this observation in mediating the axonal toxicity of paclitaxel is unclear.

Nevertheless, since ethoxyquin could potentially displace paclitaxel from Hsp90 and prevent its axonal toxicity, we investigated this possibility using a fluorescence-quenching assay. As shown in Figure 5c, when ethoxyquin was present at 1.5 µM concentration, there was no displacement of ethoxyquin across a wide dose range of paclitaxel suggesting that ethoxyquin and paclitaxel likely bind to different sites on Hsp90.

Since these findings indicated that ethoxyquin was acting through Hsp90 by modulating another activity, we evaluated the possibility that ethoxyquin might be altering its chaperone function. In order to find out which proteins that normally bind to Hsp90 are displaced by ethoxyquin, we prepared a column in which His-tagged Hsp90 was bound and treated with either ethoxyquin 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 three proteins were displaced from binding to Hsp90 by pre-treatment with ethoxyquin: ATXN2 (ataxin-2), p54/nrb (a RNA recognition domain-containing protein) and SF3B2 (splicing factor 3b, subunit 2) (Supplemental Figure 5).

In order to confirm changes in levels of these proteins in the presence of ethoxyquin, we treated DRG neuronal cell lines for 24 hours with and without paclitaxel and/or ethoxyquin and measured protein levels by Western blotting (Figure 6). As shown in Figure 6A, there was no effect of paclitaxel alone on the levels of all three proteins, but incubation of DRG neuronal cells with ethoxyquin (with
or without PTX) resulted in a dose-dependent reduction in levels of all three proteins. We then confirmed this interaction between ethoxyquin, Hsp90 and three of its client proteins in a binding assay using resin-bound His-Hsp90 and recombinant ATXN2, p54/nrb and SF3B2 (Figure 7). When this column was pre-incubated with ethoxyquin, binding of all three recombinant proteins were inhibited in a dose-dependent manner.

Although these observations led us to think that neuroprotection by ethoxyquin was mediated through hsp90 by lowering the cellular levels of these three proteins, the inactive analogues of ethoxyquin allowed us to ask which one of these three client proteins of Hsp90 was truly relevant for neuroprotection by ethoxyquin. We repeated the same experiment as outlined above (Figure 7) with derivative 2 of ethoxyquin in place of ethoxyquin and found that levels of ATXN2 and SF3B2 were not altered in the presence of EQ-der.2 suggesting that one or both of these proteins is likely to play a key role in mediating the neuroprotective effects of ethoxyquin (Figure 8). Combined together, these findings indicated that ethoxyquin might act as a modulator of chaperone activity of Hsp90 and provide neuroprotection.

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

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4,trimethylquinoline) is a synthetic antioxidant first approved by Federal Drug Administration for use in animal food almost 50 years ago. Later is was found that mice fed diet supplemented with ethoxyquin were found to live longer than littermates \(^{19}\). Other studies suggested that ethoxyquin and similar anti-oxidants may inhibit chemical carcinogenesis.
(reviewed in 19,20). However, these observations were never carried out to human studies and the primary use of ethoxyquin is still as a supplement in animal food.

Although earlier studies focused on antioxidant properties of ethoxyquin, because we started with an unbiased phenotypic screen we initially had no idea about ethoxyquin’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 ethoxyquin, which is in the micro- to milli-molar range 21,22. We again used an unbiased approach to identify proteins that interacted with ethoxyquin and found that ethoxyquin 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” for protein integrity in the cell 23,24. They do this by acting as chaperones for their client proteins; they help stabilize partially unfolded proteins, transport them across membranes within a cell, and 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, making up to approximately 1% to 2% of total protein in unstressed cells. This percentage increases to 4% to 6% of 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 it’s client proteins play critical roles in oncogenesis and metastasis (reviewed in 25). In addition, Hsp90 has been implicated in the pathogenesis of various neurodegenerative diseases and will likely be a target for disease modifying therapies 15,26. It was rather spurious that we identified Hsp90 as the binding partner for ethoxyquin 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.

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

Of these two 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 (SCA2)\(^27\). Furthermore, ataxin-2 is abnormally localized in spinal cord motor neurons of amyotrophic lateral sclerosis (ALS) patients and intermediate-length polyglutamine expansions in ataxin-2 gene are associated with increased risk of ALS\(^28\). How ataxin-2 mediates neurotoxicity and how reductions in ataxin-2 levels are involved in neuroprotection are 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 ethoxyquin is also involved in RNA processing as a component of splicing complex SF3B\(^29\). Which one of these two Hsp90 client proteins is responsible for the actual neuroprotection by ethoxyquin 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\(^30\). 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 of frontotemporal lobar degeneration or motor neuron diseases\(^31-33\). 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. In fact, when we tested a traditional Hsp90 inhibitor, 17-NAAG that is in clinical trials for cancer therapy or 17-DMAG, we did not find any neuroprotection against paclitaxel induced neurotoxicity in DRG neuronal cell line (Supplemental Figure 6). Another inhibitor of C-terminal activity of Hsp90 was recently identified as potential therapy for diabetic neuropathy. 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 paclitaxel-induced peripheral neuropathy models, but in preliminary studies we have seen that ethoxyquin does provide neuroprotection against diabetic neuropathy in a model of type-1 diabetes induced with streptozotocin (data not shown).

Although we believe ethoxyquin and analogues or other chemicals that act on Hsp90 chaperone activity may be effective in preventing distal axonal degeneration seen in paclitaxel-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 ethoxyquin may not be seen in more severe or chronic models of paclitaxel-induced peripheral neuropathy. Furthermore, how our observations with Hsp90 chaperone activity link to previously published work demonstrating neuroprotective effects of erythropoietin, calpain inhibition, WldS (Wallerian degeneration slow) mutation, alpha-lipoic acid, acetyl-L-carnitine, olesoxime or glutamate decarboxypeptidase inhibition remains to be investigated. In our study, reducing levels of Hsp90 protein itself had a partial neuroprotective effect (Figure 4) making it difficult to appreciate the full effect of reduction of Hsp90 levels on...
neuroprotection mediated by ethoxyquin. It is possible that reduced levels of Hsp90 led to diminished processing of its client proteins and reduced their levels similar to what is achieved by binding of ethoxyquin 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. Ethoxyquin and its novel analogues may prove to be a new class of drugs for neuroprotective therapy for peripheral neuropathies.

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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 Supplement.

Cell culture and in vitro neuroprotection assays

Cultures of DRG neuronal cell line and neuroprotection assays were carried out as previously described. Conditions for culturing the 50B11 DRG neuronal cells and measuring ATP levels were optimized for the 96-well plate format. Briefly, 3500 cells/well in media (Neurobasal medium, 5 µg/ml blasticidin, 10% fetal bovine serum (FBS), 0.5 mM 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) in a culture medium with reduced serum (0.2%). In the initial screening of the Spectrum library, drugs were dissolved in DMSO and diluted to the final concentration of 10 µM in the culture medium. Paclitaxel (PTX) (100 nM), capsaicin (50 µM) or zalcitabine (10 µM) were used to induce neurotoxicity. Compounds from the Spectrum library were added to the cells with one of the toxic compounds. Twenty-four hours later, cellular ATP levels were measured using ViaLight Plus kit (Cambrex) according to manufacturer’s protocol on LMax (Molecular Devices).

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 percent neuroprotection were 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 more than 50% neuroprotection were varied.
In order to validate the effect of EQ on actual neurite degeneration induced by PTX, measurements of axonal lengths were done as a previously published \(^42\). 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 hour in media (Neurobasal medium, 50 mM PS, 0.2% fetal bovine serum (FBS), 0.5 mM glutamine, 1×B-27 supplement, 0.2% glucose, 10 ng/mL glial cell line–derived neurotrophic factor). PTX, EQ and its derivatives, or vehicle controls were added to the wells for another 24 h 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 by the published paper \(^42\).

To evaluate the impact of EQ on paclitaxel's ability to kill breast cancer cells, conditions for culturing four cancer cell lines and measuring the ATP levels were optimized for the 96-well plate format. Briefly, 1500 cells/well in media (MDA-MB-231 in DMEM with 10%FBS; MCF-7 in DMEM with 10% FBS; TATD in RPMI with 10% FBS; SUM159 in DMEM/F-12 (250mL/250mL) with 5%FBS, 500 µL of 10mg/mL insulin and 25 µL of 10 mg/mL hydrocortisol) were plated in 96-well plates for 24 h. Constant concentrations of Paclitaxel (PTX) with or without EQ were added to the wells for another 24 h. Cellular ATP levels were measured using ViaLight Plus kit (Cambrex) according to manufacturer's protocol on LMax \(\Box\) (Molecular Devices).

All validation experiments and experiments involving primary DRG neurons or cancer cells were performed in triplicates and repeated at least twice. Statistical analysis was done using ANOVA. Correction for multiple comparisons was completed with Fisher’s 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 paclitaxel (25 mg/kg) on days 1, 3 and 5 as described previously. Vehicle control (saline) or different doses of ethoxyquin were administered on a daily basis by intraperitoneal administration starting on day 1 with the first dose of paclitaxel (n=10 animals per group). Two days before the start of the study, baseline thermal sensation and tail sensory nerve electrophysiology was carried out. Average paw withdrawal latency was recorded using IITC Analgesia Meter according to 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 5 cm distally. Sensory nerve action potential amplitude was recorded as average of 20 stimulations and conduction velocity was calculated. Two weeks after the last dose of paclitaxel (i.e. 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-mm punch biopsies of the foodpads were fixed in PLP fixative, and then stained with a pan-axonal marker anti-pgp antibody (Biogenesis, Cat# 7863-0504). Number of intraepidermal nerve fibers were counted in 6-10 sections for each animal and average density calculated as previously described. The study was repeated once more as an independent validation of the findings from the first study.

In order to examine the effect of ethoxyquin on paclitaxel’s ability to reduce tumor burden in vivo, we used a mouse model in which breast cancer cell line SUM-159 (3 x 10⁶ tumor cells suspended in phosphate buffered saline in a final volume of 0.15mL) was injected subcutaneously into adult male nude mice. When the tumor size reached 5 mm in diameter, the animals were randomly assigned to paclitaxel or paclitaxel with ethoxyquin groups. Paclitaxel was administered at a dose of 20 mg/kg intravenously on days 1, 4 and 8 and ethoxyquin was given as intraperitoneal administration on a daily basis for 3 weeks. At the end of three 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 plate in culture medium (Neurobasal medium, 10% fetal bovine serum (FBS), 0.5 mM glutamine, 1×B27 supplement, 0.2% glucose) without antibiotics overnight and allowed to reach 50-70% confluence. On the second day, 1 pmol siRNA (Ambion) oligomer was diluted in 250 µL Opti-MEM media, and 5 µL Lipofectamine 2000 (Invitrogen) in another 250 µL 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, 1.5 mL Opti-MEM media was added and gently mixed. In four hours after the transfection, culture media was replaced by culture media containing blasticidin (5 µg/ml), and cells were grown for 72 hours.

Protein isolation, Protein Electrophoresis (SDS-PAGE) 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 (ThermoScientific). The measurements of total protein concentrations were performed using BCA kits (ThermoScientific) on SPECTRAmax 340PC (Molecular Devices). Samples (30 µg of total protein per well) and standards (SeeBlue® Plus2 Pre-Stained Standard, Invitrogen) were loaded onto the wells (ready gel® 4-15% Tris-HCl, 50 µL-1gel; Biorad). The gel was run at 100V for 1.5h, and then transferred to PVDF membrane (Biorad) according to manufacturer’s protocol. The membrane was blocked at room temperature for 1 h in a blocking buffer (5 % milk), then incubated with primary antibody (Abcam or StressMarq) at 1:1000 dilution at 4°C overnight (please see Supplement for full details of all antibodies and catalogue numbers. This was followed by three washes and incubation with the second antibody at 1:1000 dilution at room temperature for 1 h. After three washes and development
in ECLTM Western Blotting Detection Reagents (GE-Healthcare, 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 \(^{17}\). On the day of use, the malachite green reagent was prepared. It contained malachite green (Sigma (0.0812%, w/v)), polyvinyl alcohol (Sigma (2.32%, w/v), ammonium molybdate (Sigma (5.72%, w/v, in 6 M 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 h at room temperature, becoming ready for use. The assay buffer (pH 7.4) was made by 100 mM Tris-HCl, 20 mM KCl, 6 mM MgCl\(_2\). Test compounds were dissolved in 100% (v/v) DMSO to give a stock concentration of 10 mM. This solution was diluted to six appropriate concentrations with test compounds in the assay buffer. Five µl of each compound solution was added to each well of the Perkin-Elmer 96-well assay plate. The first two 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 2.5 mM, which was placed on ice. A 10-µl ATP solution was added to each well to give a final assay concentration of 1 mM. Just before use, recombinant Hsp90 protein (StressMarq) was place on ice and suspended in cold assay buffer to make a stock solution with a concentration of 0.50 mg/ml. 10 µl of the stock Hsp90 solution was added to each well (except for the background wells that received 10 µl of assay buffer), giving a final assay volume of 25 µl. The plates were shaken (approximately 5 min) using a plate shaker (VWR DV-150 Waver) sealed with plastic film and incubated at 37 °C for 3 hours. To stop the incubation, 50 µl of the malachite green reagent was added to each well and the plate was shaken again. After addition of 10 µl of 34% sodium citrate to each well, the plate was shaken again and left to stand at room temperature for 20 min. The absorbance at 620 nM 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 SpectraMax Gemini XS plate reader (Molecular Devices) using a 96-well black PCR plate (Thermo scientific). Compounds (10mM in ethanol) were diluted with 50mM Tris-HCl (pH 7.4) to obtain a solution with a 15 µM final concentration. Aliquots of proteins were added to the compounds solution and mixed with micro pipette by sucking the solution gently up and down, then fluorescence intensities ($\lambda_{\text{ex}} = 350$ nM, $\lambda_{\text{em}} = 450$ nM) were measured at 25 °C according to published methods. Equilibrium dissociation constant $K_d$ was obtained by fitting the data using nonlinear least squares option of GraphPad Prism software. All experiments were completed in duplicates and repeated at least three times.

**Binding studies using affinity capture chromatography**

The AminoLink Plus Immobilization Kit was purchased from Thermo Scientific. The assay procedure was based on a published paper and was carried out according to manufacturer's protocol. AminoLink Plus Coupling resin was incubated overnight in a solution of 200 µmol EQ-der. 6 (DMSO: pH 7.2 Coupling buffer / 20:80). Then the resin was equilibrated to pH 7.2 in phosphate buffer containing 150 mM NaCl. To characterize the compound linked to AminoLink, we used HPLC 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 50 mM sodium cyanoborohydride solution (40 uL in 2 mL 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 20 grams by unilateral excision of the L3, L4, L5, and L6 dorsal root ganglia) were passed through these affinity columns and extensively washed to remove non-specifically bound proteins. Elution was performed with 1 mM EQ-der.6 in elution buffer. The elute was resolved by SDS-PAGE, and then revealed by Pierce SilversStain and Coomassie Brilliant Blue. Bands of interest were cut out of the gel and analyzed by
nano-LC ESI/MS/MS Orbitrap Velos FTFT. 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 (Thermoscientific) according to the manufacturer's specifications. Half of Hsp90ab1 Coupling resin was incubated at 4 °C for 3 h in a solution of 0.5 mg/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 h and extensively washed with 10mM pH 7.5 Tris-HCl to remove non-specifically bound proteins. After additional washing with 10 mM pH 7.5 Tris-HCl containing 500 mM NaCl, elution was performed with 400 µL 100 mM Glycine (pH 2.8). Elutes were resolved by SDS-PAGE gel and then revealed by Pierce SilversStain. 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 three client proteins**

Recombinant Hsp90ab1-His (Abcam) was linked to His Pur TM Ni-NTA resin (Thermoscientific) according to the manufacturer's specifications, then incubated at 4 °C for 2 h with various concentrations of EQ or inactive analogue, EQ-Der.2 (EQ=0, 10nM, 30nM, 100nM, 300nM, 1000nM in ethanol with protein inhibitor). After several washings, recombinant ATXN2, SF3B2 and p54/nrb proteins (Novus Biologicals) 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 non-specifically bound proteins. The beads were incubated in 100 µL loading buffer at 95 °C for 5 minutes, then the buffer containing protein were resolved by SDS-PAGE gel and revealed by Western blotting.
Figure Legends

Figure 1: Neuroprotection by ethoxyquin in vitro
(A) 50B11 DRG neuronal cells were differentiated, then exposed to paclitaxel (PTX) with or without various concentrations of EQ. ATP levels were measured after 24 h. (B) Primary rat DRG neurons were grown in culture and allowed to extend their axons for 24 h. Then, they were exposed to PTX or EQ for another 24 h. Cells were fixed, 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)

Figure 2: Neuroprotection by ethoxyquin against paclitaxel induced peripheral neuropathy
Co-administration 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).

Figure 3: Ethoxyquin does not block paclitaxel's chemotherapeutic effects
When four different breast cancer cell lines (A-D) were grown in culture, PTX reduced cell viability by 35-75%; 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 paclitaxel's ability to reduce tumor size (E) or tumor weight (F). (n= 5 per group)

Figure 4: Loss of neuroprotection by RNAi of Hsp90
When Hsp90 protein levels were reduced by siRNA, neuroprotection provided by EQ against toxicity of 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)

**Figure 5: Binding of ethoxyquin to Hsp90**
(A) EQ binds to recombinant Hsp90 with Kd of 280 nM using fluorescence quenching method. (B) EQ does not alter the ATPase activity of hsp90 across a wide dose range. (C) EQ does not alter the binding of paclitaxel to recombinant Hsp90.

**Figure 6: The effect of ethoxyquin on three candidate client proteins of Hsp90**
Representative Western blots of each candidate protein in 50B11 DRG neuronal cultures when treated with PTX alone (A), EQ alone (B) or 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 three separate experiments per condition).

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

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

Figure 1: Neuroprotection by ethoxyquin in vitro (A) 50B11 DRG neuronal cells were differentiated, then exposed to paclitaxel (PTX) with or without various concentrations of EQ. ATP levels were measured after 24 h. (B) Primary rat DRG neurons were grown in culture and allowed to extend their axons for 24 h. Then, they were exposed to PTX or EQ for another 24 h. Cells were fixed, 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)

90x38mm (300 x 300 DPI)
Figure 2: Neuroprotection by ethoxyquin against paclitaxel induced peripheral neuropathy
Co-administration 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).
Figure 3: Ethoxyquin does not block paclitaxel’s chemotherapeutic effects
When four different breast cancer cell lines (A-D) were grown in culture, PTX reduced cell viability by 35-75%; 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 paclitaxel’s ability to reduce tumor size (E) or tumor weight (F). (n= 5 per group)

264x357mm (300 x 300 DPI)
Figure 4: Loss of neuroprotection by RNAi of Hsp90

When Hsp90 protein levels were reduced by siRNA, neuroprotection provided by EQ against toxicity of 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)
Figure 5: Binding of ethoxyquin to Hsp90
(A) EQ binds to recombinant Hsp90 with Kd of 280 nM using fluorescence quenching method. (B) EQ does not alter the ATPase activity of hsp90 across a wide dose range. (C) EQ does not alter the binding of paclitaxel to recombinant Hsp90.

231x524mm (300 x 300 DPI)
Figure 6: The effect of etoxyquin on three candidate client proteins of Hsp90

Representative Western blots of each candidate protein in 50B11 DRG neuronal cultures when treated with PTX alone (A), EQ alone (B) or 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 three separate experiments per condition).

163x90mm (300 x 300 DPI)
Figure 7: The effect of ethoxyquin on binding of three candidate client proteins to recombinant Hsp90
(A) Representative Western blots of three candidate client proteins bound to recombinant Hsp90-his column
when incubated with various concentrations of EQ. Bar graphs denotes averages of 3 experiments for ATXN2
((B), SF3B2 (C) and p54/nrb (D).
Figure 8: The effect of EQ-der.2 (inactive analogue of EQ) on binding of three candidate client proteins to recombinant Hsp90
(A) Representative Western blots of three candidate client proteins bound to recombinant Hsp90-his column when incubated with various concentrations of EQ-der.2. Bar graphs denotes averages of 3 experiments for ATXN2 ((B), SF3B2 (C) and p54/nrb (D).
Supplement

Figure Legends

Supplemental Figure 1: Chemical structures of ethoxyquin and its derivatives

Supplemental Figure 2: Neuroprotection assays using derivatives of EQ
50B11 DRG neuronal cells were differentiated and then exposed to paclitaxel (PTX) with or without various concentrations of EQ derivatives. ATP levels were measured after 24 h. (n=8-10 per group; * = p<0.05 compared to PTX alone)

Supplemental Figure 3: Identification of proteins that bind to EQ

(A) SDS-Page analysis of proteins eluted from EQ-der.6 column (silver stained). Stars mark the bands from rat DRG and 50B11 rat DRG neuronal cell lysates that were excised and analyzed by mass spectrometry.

(B) Representative images of Western blots of 50B11 DRG neuronal cultures that were treated with siRNA constructs. Levels of the proteins identified by mass spectroscopy were downregulated to varying degrees; GAPDH siRNA served as a positive control for RNAi (ANXA2: annexin A2; HNRNPA3: similar to heterogeneous nuclear ribonucleoprotein A3 isoform 3; ACADL: acyl-Coenzyme A dehydrogenase, long-chain; PSMC6: similar to proteasome 26S ATPase subunit 6; PHGDH: 3-phosphoglycerate dehydrogenase; ATPα: ATP synthase alpha subunit precursor; ALDH3A2: aldehyde dehydrogenase family 3, subfamily A2; HSP90AB1: heat shock 90kDa protein 1, beta isoform 2). Note we were unable to reduce protein levels of ACADL and maintain cell viability despite trial of multiple siRNA constructs.
Supplemental Figure 4: Effect of RNAi on EQ mediated neuroprotection
When protein levels were reduced by siRNA, neuroprotection provided by EQ against toxicity of PTX was unchanged for all of the above 6 proteins. (n=8-10 per group)

Supplemental Figure 5: Identification of proteins whose binding to Hsp90 was modulated by EQ
(A) SDS-Page analysis of proteins eluted from Hsp90-His column with EQ (sample) or without EQ (control) after a lysate of 50B11 DRG neuronal cells were passed through the column (silver stained). Stars mark the bands that were excised and analyzed by mass spectrometry.

Supplemental Figure 6: Effect of hsp90 ATPase activity inhibitors on paclitaxel induced toxicity in 50B11 DRG neuronal cells
50B11 DRG neuronal cells were differentiated, and then exposed to paclitaxel (PTX) with or without various concentrations of Hsp90 ATPase inhibitors, 17-NAAG or 17-DMAG. ATP levels were measured after 24 h. (n=8-10 per group)
Supplemental Methods

List of primary antibodies

- Anti ANXA2 antibody ab41803(Abcam)
- Anti HNRNPA3 antibody ab94593(Abcam)
- Anti ACADL antibody ab82853(Abcam)
- Anti PSMC6 antibody ab22639(Abcam)
- Anti PHGHD antibody ab57030(Abcam)
- Anti ALDH3A2 antibody ab72774(Abcam)
- Anti Hsp90 antibody ab13492(Abcam)
- Anti ATPα antibody MS502(Mitosciences)
- Anti GAPDH antibody cat#AM4300 (Ambion)
- Anti ATXN2 antibody ab72263(Abcam)
- Anti P54/nrb antibody ab45359(Abcam)
- Anti SF3B2 antibody sc-101133(Santa Cruz)
- Anti-His(c-Term)-HRP Ab Lot no. 839540(Invitrogen)
- Anti GST antibody ab9085(Abcam)
- Anti β-actin antibody ab3280 (Abcam)

List of siRNA constructs

- Silencer® Select GAPDH siRNA (Hs, Mm, Rn) cat.4390849(Ambion)
- Hsp90ab1 siRNA cat.S235784(Ambion)
- HNRNPA3 siRNA cat.S168604, cat. S188733(Ambion)
- ANXA2 siRNA cat.S132715(Ambion)
- ACADL siRNA cat.S129378, cat. S129377(Ambion)
- PHGHD siRNA cat.S132892, cat. S132891(Ambion)
ALDH3A2 siRNA cat. S134730, cat. S134732(Ambion)

ATPα siRNA cat.S134814(Ambion)

PSMC6 siRNA cat.XM-214147(sigma)

**List of Recombinant Proteins**

- Ataxin-2 partial Recombinant protein H00006311-Q01 (Novus)
- SF3B2 Recombinant protein H00010992-P01(Novus)
- NONO Recombinant protein H00004841-Q01(Novus)
- Hsp90 protein(His tag) ab80353(abcam)

**Synthesis protocols for novel derivative of EQ (EQ-Der.6)**

**General experiment.** Chemicals were purchased from Sigma-Aldrich. Solvents were purchased from suppliers as anhydrous grade. NMR spectra were recorded at room temperature on Bruker-400 MHz spectrometer. Chemical shifts are reported in ppm with TMS as the internal standard.

![Chemical structure](image)

**Boc-Der.6:** To 2, 2, 4-trimethyl-1, 2-dihydro-6-quinolinol (94 mg, 0.5 mmol) in anhydrous DMF (2 mL) cooled to -20 °C was added solid NaH (14 mg, 0.63 mmol) in four portions. After 5 min stirring, 5-(t-Boc- amino)-1-pentyl bromide (140 mg, 0.53 mmol) in DMF (1 mL) was slowly added into above solution. The resulted reaction mixture was kept stirring at r.t. for 1 hour then check NMR. NaH residue was quenched with MeOH (0.1 mL), volatile material was removed under reduced pressure and residue was purified.
with flash chromatography with 4:1 then 3:1 Hexane: Acetone as elute. Pure product Boc-Der.6 (150 mg, 0.4 mmol) was obtained as light yellow oil in a 80% yield.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.67 (m, 1H), 6.57 (m, 1H), 6.39 (m, 1H), 5.35 (s, 1H), 4.63 (b, 1H), 3.87 (t, J=3.83, 2H), 3.13 (m, 2H), 1.96 (s, 3H), 1.74 (m, 2H), 1.51 (m, 4H), 1.44 (s, 9H), 1.24 (s, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 155.96, 151.35, 137.45, 129.62, 128.50, 122.90, 114.29, 113.60, 110.92, 78.95, 51.67, 40.50, 30.31, 29.85, 29.16, 28.43, 23.40, 18.58. Mass:M$^+$ 374.25608 (calculated 374.25694)

**Der.6:** To Boc-Der.6 (30 mg, 0.08 mmol) in CH$_2$Cl$_2$ (1 mL) was added TFA (0.2 mL) and color of the reaction mixture immediately changed from brown to green. Keep stirring for 2 h then volatile material was removed under vacuum. Crude material was purified via column chromatography with 1:10 MeOH: CH$_2$Cl$_2$ as elute. Pure product Der.6 (20 mg, 0.076 mmol) was obtained as a yellow oil in a 95% yield. Product was extracted with CHCl$_3$ and pH=14 water before NMR analysis. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.68 (d, J=2.73, 1H), 6.58 (dd, J1=8.47, J2=2.76, 1H), 6.40 (d, J=8.44, 1H), 5.36 (s, 1H), 3.89 (t, J=6.47, 2H), 2.72 (t, J=6.16, 2H), 1.97 (s, 3H), 1.76 (m, 2H), 1.51 (m, 6H), 1.25 (s, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 151.44, 137.44, 129.63, 128.57, 122.98, 114.31, 113.60, 111.00, 68.68, 51.71, 42.11, 33.49, 30.35, 29.37, 23.45, 18.60. Mass:M$^+$ 274.20462 (calculated 274.20451)
Supplemental Figure 1

- **EQ**
- **O-acetyl hydroquin.** (Der.1)
- **O,N-actyl hydroquin.** (Der.-2)
- **N-acetyl hydroquin.** (Der.3)
- **Hydroquin.** (Der.4)
- **Quin.** (Der.5)
- **O-aminopentyl hydroquin.** (Der.6, EQ-6)
- **O-aminopentyl hydroquin.** chloride (Der.6 Cl salt)
- **O-pentyl hydroquin.** (Der.7, EQ-7)

**Compounds:**
- **EQ**
- **O-acetyl hydroquin.**
- **O,N-actyl hydroquin.**
- **N-acetyl hydroquin.**
- **Hydroquin.**
- **Quin.**
- **O-aminopentyl hydroquin.**
- **O-aminopentyl hydroquin.** chloride
- **O-pentyl hydroquin.**

**Chemical Structures:**
- 6-ethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline
- 2, 2, 4-trimethyl-1, 2-dihydroquinolinol-6-y1 acetate
- 1-acetyl-2, 2, 4-trimethyl-1, 2-dihydroquinolinol-6-y1 acetate
- 1-acetyl-2, 2, 4-trimethyl-1, 2-dihydroquinolinol-6-o1
- 2, 2, 4-trimethyl-1, 2-dihydro-6-quinolinol
- 2, 6-Dihydro-2, 2, 4-trimethyl-6-quinolone
- 6-(5-amino)-pentox-2,2,4-trimethyl-1,2-didroquinolin e
- 6-pentoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline
- 6-(2-amino)-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline chlorid
Supplemental Figure 3

A. Seeblue sample blank nerve 50B-11 DRG SDS-PAGE MW (KDa)

B. GAPDH ANXA2 HNRNPA3 ACADL PSMC6 PHGDH ATPa ALDH3A2 Hsp90 ab1

C. Integrated density % (siRNA/protein)

- GAPDH ANXA2 HNRNPA3 ACADL PSMC6 PHGDH ATPa ALDH3A2 Hsp90 ab1

siRNA protein actin
Supplemental Figure 5
Supplemental Figure 6

**17-NAAG**

Relative ATP level

- Control
- PTX (100 nM)
- PTX+17AAG (100 nM)
- PTX+17AAG (300 nM)
- PTX+17AAG (3000 nM)
- PTX (100 nM) + 17AAG (100 nM)
- PTX (100 nM) + 17AAG (300 nM)
- PTX (100 nM) + 17AAG (3000 nM)

**17-DMAG**

Relative ATP level

- Control
- PTX (100 nM)
- PTX+17DMAG (100 nM)
- PTX+17DMAG (300 nM)
- PTX+17DMAG (3000 nM)
- PTX (100 nM) + 17DMAG (100 nM)
- PTX (100 nM) + 17DMAG (300 nM)
- PTX (100 nM) + 17DMAG (3000 nM)